Investigating the potential antiproliferative effect of *Moringa oleifera* aqueous leaf extract in MCF-7 breast cancer cells

by

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24 November 2023

Declaration

I hereby declare that this work, submitted for the degree Master of Medical Science with specialisation in Physiology at the University of the Free State, is my own original work and has not previously been submitted, for degree purposes or otherwise, to any other institution of higher learning. I further declare that all sources cited or quoted are indicated and acknowledged through a comprehensive list of references. Copyright hereby cedes to the University of the Free State.

Malebogo M. Moremane	24 November 2023
Name and Surname	Date

Dedication

I DEDICATE THIS DEGREE TO MYSELF FOR ALL THE HARD WORK AND PERSEVERENCE AND TO MY DAUGHTER FOR BEING MY BIGGEST REASON TO WORK EVEN HARDER.

Organisation of the thesis

The current thesis comprises six chapters, with the introduction being the first chapter. The introduction describes cancer as a non-communicable disease and outlines the incidence globally. The introduction also identifies the research problem statement and describes the approach to the study to mitigate these challenges. The literature review is the second chapter of this study, outlining the epidemiology, aetiology, pathophysiology and diagnosis of breast cancer. Additionally, the classification, stages and grades of breast cancer are addressed. Chapter 3 outlines the methodology followed to conduct the study to test the hypothesis. The third chapter also presents the design of the study, the type of breast cancer cells used, materials utilised, and procedures conducted in this study, including data analysis, laboratory techniques, reliability, and validity, as well as ethical considerations. In the fourth chapter, the results obtained from the experiments are presented. In Chapter 5, the findings pertaining to this study are discussed in detail and incorporated with reference to the existing body of literature available. Chapter 6 concludes the study, discussing the limitations and recommendations for future studies.

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Presentations

Investigating the potential antiproliferative effect of *Moringa oleifera* aqueous leaf extract in MCF-7 breast cancer cells

The findings of the above research study were presented at the following research events:

1) MedTx presentation at the University of the Free State, Department of Basic Medical Sciences, Faculty of Health Sciences. 19 August 2022.
2) M.Med.Sc Project presentations at the University of the Free State, Department of Haematology and Molecular Cell Biology, Faculty of Health Sciences. 02 May 2023.
3) Faculty Research Forum at the University of the Free State (Faculty of Health Sciences). 24 August 2023.
4) MedTx presentation at the University of the Free State, Department of Basic Medical Sciences, Faculty of Health Sciences. 29 September 2023.

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List of abbreviations

ANOVA	One-Way Analysis of Variance
Apaf-1	Apoptotic protease-activating factor 1
ATC	Anthracyclines
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BSA	Bovine Serum Albumin
CVD	Cardiovascular Disease
CCM	Complete culture media
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
FBS	Fetal bovine serum
GSH	Glutathione
HAUSP	Herpesvirus-associated ubiquitin-specific protease
kDa	Kilodalton(s)
Keap-1	Kelch-like epichlorohydrin-associated protein 1
MCF-7	Michigan Cancer Foundation-7
Mdm2	Mouse double minute 2 homolog
Mdmx	Murine double minute X
MnSOD	Manganese superoxide dismutase
MO	*Moringa oleifera*
MTT	Methyl thiazoltetrazolium
NADPH	Nicotinamide adenine dinucleotide phosphate
Nrf2	Nuclear factor erythroid 2-related factor 2
PARP-1	Poly (ADP-ribose) polymerase 1
PPE	Personal Protective Equipment
Redox	Oxidation-reduction
qPCR	Quantitative Polymerase chain reaction
RLU	Relative light unit

RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SA	South Africa
SD	Standard deviation
SOD	Superoxide dismutase
TTBS	Tris-buffered saline 0,05% tween-20
US	United States
USA	United States of America
WHO	World Health Organization

Abstract

Background: Breast cancer is associated with elevated mortality and morbidity rates in women across the world. Current chemotherapeutic drugs such as Doxorubicin (Dox) display contra-indications, thus expressing the need for alternative treatment methods. Therefore, to reduce the cancer burden, the study's objective was to investigate whether an aqueous leaf extract of *Moringa oleifera* (MO), a medicinal tree native to India and indigenous to Africa, possesses antiproliferative potential against MCF-7 breast cancer cells.

Methodology: In order to suppress cell growth, MCF-7 cells were treated with MO (2600 µg/ml) for 72 hours. Cells were also co-exposed with Dox (0.978 µM), modelled as a positive control. The unexposed cells served as the control. Biochemical analysis was conducted after 72 hours (MTT, GSH, DCFH-DA, ATP, Caspase 3/7, 8/9, qPCR and western blot assays) to assess the efficacy of MO and Dox.

Results: *Moringa oleifera* aqueous leaf extract significantly reduced the proliferation of breast cancer cells by inducing oxidative stress through increasing ROS whilst decreasing glutathione content and Nrf2 protein expression. Additionally, MO induced apoptosis by increasing caspases -3/7, -8, -9, metabolic activity and upregulating p53. Similar results were observed in Dox-exposed cells. Furthermore, cell death due to MO was activated with downregulation of Bcl-2, PARP-1 and Bax. Dox decreased the growth of breast cancer cells by increasing ROS. In contrast, Dox induced chemoresistance through increased GSH content and downregulated apoptotic protein Bax and p53 gene. However, the MO + Dox combination induced antiproliferative potential similarly to MO, suggesting a possible synergistic effect.

Conclusion: MO aqueous leaf extract displayed antiproliferative potential by inducing apoptosis and oxidative damage to the MCF-7 breast cancer cells

Keywords: Apoptosis, breast cancer, oxidative stress, antiproliferative, *Moringa oleifera*

CHAPTER 1

INTRODUCTION

Non-communicable diseases signify almost 71% of worldwide mortalities, with cancer and cardiovascular diseases accounting for 9,3 and 17,9 million deaths, respectively (World Health Organization 2021a). Cancer is a condition in which some of the body's cells proliferate uncontrollably and has been observed to have the ability to metastasise in various body parts (World Health Organization 2021a). Additionally, it can regenerate in different sides of the human body and is made up of trillions of cells. Human cells have a lifespan and are replaced through cell division to form new cells as the body needs them (Institute 2021b). When there is ageing or injury to the cells, apoptosis takes place (which can also be triggered in a caspase dependent manner). Following that, the regeneration of new cells occurs. Sometimes, this regulated process malfunctions, allowing abnormal cells to continue to grow (Institute 2021b). These abnormal cells may form localised primary tumours (World Health Organization 2021a).

Tumours may form cancerous or non-cancerous tissue (Ohshika et al. 2021). Cancerous tissue is malignant, whereas non-cancerous is benign (Ohshika et al. 2021). Malignant tumours can migrate to nearby tissues and travel to distant sites in the body to form new tumours; this process is called metastasis. Moreover, there are various malignancies which form solid tumours. However, blood cancers, such as leukaemia, generally do not.

Nevertheless, when cancerous tumours are removed, they usually do not grow back, whereas in other cases, they do. On the other hand, benign tumours remain localised and can sometimes have a large mass and become life-threatening (Institute 2021a). This may cause crowding of the normal structures inside the skull, leading to benign tumours in the brain (Institute 2021a; Fayed 2021). Additionally, non-cancerous tumours can pose a threat to one’s health by pressing on vital organs (Fayed 2021). Fayed also noted that the non-cancerous tumours do not invade the neighbouring tissues. In contrast, malignant tumours invade neighbouring tissues and are considered dangerous to one’s health (Fayed 2021).

The most common malignant tumour in women is breast cancer, which is the cause of the highest female mortality rate globally (Institute 2021a). Breast cancer development and progression are linked to several complicated anatomical changes and molecular processes.

The present level of knowledge regarding the normal macro and microanatomy of the human mammary gland has been addressed in the literature recently (Abrahams, 2014). Moreover, it was stated that the human mammary gland also undergoes distinct development and differentiation transformations from embryogenesis through postmenopausal age (Abrahams, 2014). However, even today, to describe the anatomy of the breast, Sir Astley Paston Cooper's (1768–1841) descriptions of mammalian breast dissections in 1840 are still cited (Abrahams, 2014). Abrahams believed that doctors need to have a good understanding of the anatomy of the breast to perform effective clinical breast exams (Abrahams, 2014). The stages of breast development include pre-puberty, puberty, pregnancy, lactation-associated remodelling, and post-lactational and post-menopausal involution. Breast tissue differentiates and becomes more specialised as the mammary gland develops into an adult and efficient milk-secretory organ during the pregnancy and lactation cycle (PLC). However, as multiparous females are susceptible to the PLC cycle, childbearing and nursing may offer some long-term protection against the onset of breast cancer (Abrahams, 2014).

Breast glandular tissue has ductal epithelium that makes up 85% of cases and lobular epithelium that makes up 15% of cases of breast cancer (Waks and Winer, 2019). The malignant tissue matures within the duct or lobule ("in situ"), where it is asymptomatic and has a decreased likelihood of proliferating (metastasis). These in situ (Stage 0) tumours may develop over time and spread to the neighbouring lymph nodes (regional metastasis), other body organs (distant metastasis), or the breast tissue itself (invasive breast cancer) (Waks and Winer 2019). Breast cancer can grow rapidly in the female population, thus resulting in increased mortality rates. According to estimates, metastasis caused 90% of cancer-related deaths (Seyfried and Huysentruyt 2013).

In most cases, treatment becomes more effective, especially when the breast cancer is prematurely discovered. Radiation therapy, hormone therapy, and surgical removal of a tumour are frequently used in conjunction to treat breast cancer (World Health Organization 2021b). Also employed include chemotherapy and/or targeted biological treatment (World Health Organization 2021b). Such therapy can stop the growth and spread of cancer, thereby saving lives (World Health Organization 2021b).

Cancer survival rates have increased due to the discovery of anti-cancer drugs such as anthracyclines (ATCs) (Lipshultz et al. 2011; McGowan et al. 2017b). Anthracyclines are frequently referred to as antibiotics and are generated from the Streptomyces bacteria

(McGowan et al. 2017b). Doxorubicin and daunorubicin are the two most important ATCs that are used therapeutically. As a kind of chemotherapy, they are used to treat several cancers, including breast cancer (McGowan et al. 2017b). These substances impede DNA metabolism-related biological processes, killing rapidly dividing cells (Johnson-Arbor and Dubey 2022). The majority of anthracyclines are injected straight into the bloodstream, and common adverse effects include hair loss, nausea, vomiting, and a reduction in immune cell production. The most serious potential adverse effect is dose-related cardiac tissue damage; however, current treatment protocols are created to minimise this risk (McGowan et al. 2017b).

Since the introduction of ATCs over 50 years ago, the survival rate of cancer in both genders has increased. Doxorubicin (Dox) is one of the ATCs used to treat various malignancies, including breast cancer (Hortobagyi 1997; Abdullah et al. 2019). However, the use of this chemotherapeutic agent, Dox, has resulted in several contraindications after its administration. The side effects of Dox may present in a matter of days (acute toxicity) or even years (chronic toxicity) following chemotherapeutic treatment. Dox-associated side effects include the development of heart disease as well as resistance to the treatment (Ji et al. 2019). Therefore, the steps that are currently taken to overcome these side effects include the investigation of alternative drugs, such as herbal drugs, as potential cancer therapeutics.

Several researchers became interested in the use of herbal medicines since they are affordable, easily accessible, natural, and have a number of significant biological qualities (Inamdar et al. 2010; Jung 2014; Sangweni et al. 2020). In the United States (US), between 50% and 60% of cancer patients use nutrition or treatments made from various plant and animal components, either exclusively or concurrently with a traditional treatment strategy such as chemotherapy and/or radiation therapy (Greenwell and Rahman 2015; Mansoori et al. 2017; Bukowski, Kciuk, and Kontek 2020). These include curcumin from turmeric, genistein from soybeans, tea polyphenols from green tea, resveratrol from grapes, sulforaphane from broccoli, isothiocyanates from cruciferous vegetables and silymarin from milk thistle (Wang et al. 2012).

Of interest in this study is *Moringa oleifera* (MO), a medicinal tree with the potential of being an anti-cancer agent, as it is an antioxidant as well as an inducer for cell death in different cancer cell lines such as $HepG_2$ liver cancer cells (Suphachai 2014). The MO tree is small in size, approximately 5 to 10 m in height. It is cultivated worldwide due to its multiple

utilities (Farooq et al. 2012). Every part of MO is used for nutritional and/or medicinal purposes. Besides being a good source of protein, vitamins, oils, fatty acids, micro-macro minerals elements and various phenolics, they have anti-cancer and anti-inflammatory properties. The tree, MO has also been shown to inhibit oxidation (leaves), act as an antibacterial agent (seeds), hepatoprotective (leaves), and ulcer-healing properties (seeds and leaves) as well as diuretic (flowers and leaves), antiurolithiatic (seeds), and antihelmintic properties (Farooq et al. 2012). MO’s multiple pharmaceutical effects are capitalised as a therapeutic remedy for various diseases in the traditional medicinal system (Farooq et al. 2012). However, there is still insufficient information on the usage of MO in breast cancer. Therefore, this study aimed to analyse the potential of MO as an antiproliferative agent, mainly focusing on its potential to increase oxidative stress and cause apoptosis in the MCF-7 breast cancer cell line.

1.2 PROBLEM STATEMENT

Breast cancer is responsible for an increased number of deaths among South Africans, specifically in women (Rayne et al. 2019). Current chemotherapeutic regimens are very expensive, limited in access, and have been proven to have several side effects, with drug resistance being a common occurrence (Bukowski, Kciuk, and Kontek, 2020; Mansoori et al. 2017). In addition, the current anti-cancer drugs are non-specific as they target both cancerous and normal healthy cells, highlighting the urgent need for alternative treatment. There is a high interest in herbal drugs, including MO, as an anti-cancer adjuvant treatment (Karim et al. 2016). Many studies have shown MO's potential through its ability to increase oxidative stress, activate apoptosis and subsequently reduce the rapid growth of cancer cells (Suphachai 2014). Investigations have been conducted on various parts of the MO tree, including the seeds and the stem. However, a clear understanding of the antiproliferative mechanisms of MO aqueous leaf extract on the MCF-7 breast cancer cells is required.

1.3 RESEARCH RATIONALE

The incidence of female deaths associated with breast cancer is increasing rapidly in South Africa (SA) (Lince-Deroche et al. 2017; Globocan, 2020). Breast cancer cells have been proven to develop resistance over the years to the currently used anti-cancer drugs (Ji et al. 2019). In addition, several side effects are associated with using some chemotherapeutic

agents (e.g., chemoresistance and cardiovascular diseases) (Ji et al. 2019). Furthermore, many approved anti-cancer drugs such as Dox are not only non-specific in their mode of action, thereby destroying and becoming cytotoxic to normal healthy cells, but are also expensive and inaccessible to poorer communities (Leighl et al. 2021). As a result, plant-based therapy has become the most recommended treatment as they are less invasive at specific concentrations and inexpensive. *Moringa oleifera* (MO), a tree native to India and Africa, is of interest in this study. Various studies have reported MO's ability to be a chemotherapeutic agent (Berkovich et al. 2013; Suphachai 2014; Abd-Rabou et al. 2017). Not only may MO potentially be used as an antiproliferative drug for malignancies due to its ability to induce oxidative stress and apoptosis, but it has been used traditionally to treat several other ailments, such as human immunodeficiency virus and diabetes mellitus type 2 (Suphachai, 2014). MO is also readily available in rural and urban areas, which facilitates its traditional medicinal use. A safe and effective dose of MO is yet to be determined. MO treatment has been explored on different cancer cell lines, including breast cancer; however, its antiproliferative potential on breast cancer cells requires further investigation. Moreover, the aqueous leaf extract of MO is used more readily in traditional medicines, and it is important to find the scientific evidence behind its use. The anti-cancer mechanism of MO aqueous leaf extract on MCF-7 cells is not yet fully elucidated. Therefore, this study aimed to investigate the potential of the MO aqueous leaf extract as an anti-cancer drug using the MCF-7 breast carcinoma cell line. A clear understanding of oxidative stress and apoptotic potential of MO in breast cancer is therefore required.

1.4 AIM OF THE STUDY

The study aimed to investigate the antiproliferative effect of MO aqueous leaf extract in MCF-7 breast cancer cells.

1.5 RESEARCH QUESTIONS

The following overarching research questions guided the study:

1. Can the administration of MO increase the production of free radicals in breast cancer cells?
2. Can cell death be induced in MO-treated breast cancer cells?

1.6 OBJECTIVES OF THE STUDY

In order to achieve the above-stated aim, the following objectives were pursued:

- Investigate whether MO aqueous leaf extract can increase intracellular reactive oxygen species (ROS) in MCF-7 cells.
- Assess whether MO aqueous leaf extract can decrease antioxidant levels in MCF-7 cells.
- Investigate whether MO aqueous leaf extract can induce apoptosis in MCF-7 cells.
- Investigate the dose-response effect of MO and Dox on MCF-7 cells.

1.7 METHODOLOGY OF THE STUDY

The current study is an experimental quantitative study (Noyes et al. 2019). All the details of the methodology are outlined in Chapter 3. The MCF-7 breast cancer cells were treated with MO and Dox to determine the potential antiproliferative effect on breast cancer cells. Tissue culture methodology and several laboratory assessments were conducted to obtain the results of the study.

1.8 CONCLUSION

This chapter provided a brief outline of MO as a potential anti-cancer drug. The aims and objectives of the study were discussed, including the rationale. A description of the methodology was also outlined.

CHAPTER 2

LITERATURE REVIEW

Chapter 2 reviews the herbal plant, MO, and its potential as a chemotherapeutic agent. The review includes MO's ability to increase oxidative stress as well as its potential to induce cell death in various cancer cell lines. However, this study focused on the MCF-7 breast cancer cell. Additionally, the mechanistic pathways MO uses to induce its antiproliferative effect were described. This study compared its possible antiproliferative effect with the current chemotherapeutic drug, Dox, as it has been proven to exert several side effects.

2.1.1 Cancer

Non-communicable diseases such as cancer contribute to high global morbidity and mortality rates (Boutayeband Boutayeb 2005; Bray et al. 2018). Cancer is described as a disease state with anomalous cell development and uncontrollable cell division (Editor and Shen 2011). Several hallmarks of cancer describe the abilities of cancer cells to permit its progression, including the ability to sustain signalling of cell proliferation, eluding growth suppressors, and cell death resistance (**Figure 2.1**) (Hanahan and Weinberg 2011; Hanahan 2022). Additionally, the induction of angiogenesis during the cancer disease state and the ability of cancerous cells to avoid destruction by immune cells are also included as the hallmarks of cancer. Moreover, the deregulation of cellular metabolism in cancerous cells and the activation of invasion and metastasis during disease progression also form part of cancer hallmarks (Hanahan and Weinberg 2011; Martin et al. 2013; Dersarkissian 2021). The hallmarks of cancer continue to be the cardinal point of understanding the microenvironment of various cancers and provide a systematic basis to target different cancers for improved therapies (De Palma and Hanahan 2012; Yu, Huang, and Zhou 2019; Iannuccelli et al. 2020).

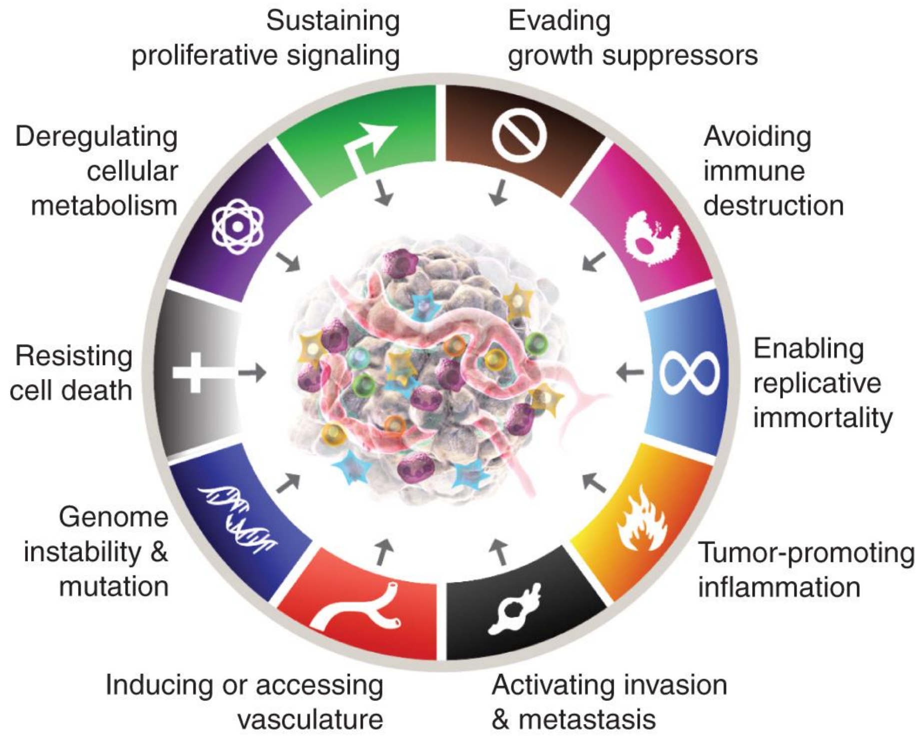

Figure 2.1: **Hallmarks of cancer**: These hallmarks aid in clearly understanding various human cancers. They include sustaining the signalling of cell proliferation, eluding growth suppressors, and cell death resistance. Additionally, induction of angiogenesis, avoiding destruction by immune cells, deregulation of cellular metabolism, genome instability, enabling of replicative immortality as well as tumour-promoting inflammation and the activation of invasion and metastasis (Hanahan and Weinberg 2011; Hanahan 2022).

2.1.2 Breast cancer epidemiology

Cancer is the second leading cause of death worldwide, with breast cancer being the most common type of cancer affecting women (Bray et al. 2018). An estimated 10 million cancer deaths were recorded globally in 2020 (Sung et al. 2021). Of the estimated 19,3 million new cancer cases reported globally, breast cancer diagnosis in women accounted for 11,7 % (Sung et al. 2021). It is predicted that more than 1,3 million untreated cases will succumb to the disease unless the necessary course of action is taken (Sung et al. 2021). South Africa (SA) has a total population of 59 million, with a reported 108 168 patients diagnosed with cancer in 2020 and 56 802 cancer-related deaths (Globocan 2020). Almost a third of the recorded cancer cases (27,1 %) accounted for breast cancer (Globocan 2020). Although the incidence

of breast cancer is widespread across the world, its mortality rate, survival rate, and prevalence vary in different parts of the world because of different associated risk factors (Momenimovahed and Salehiniya 2019).

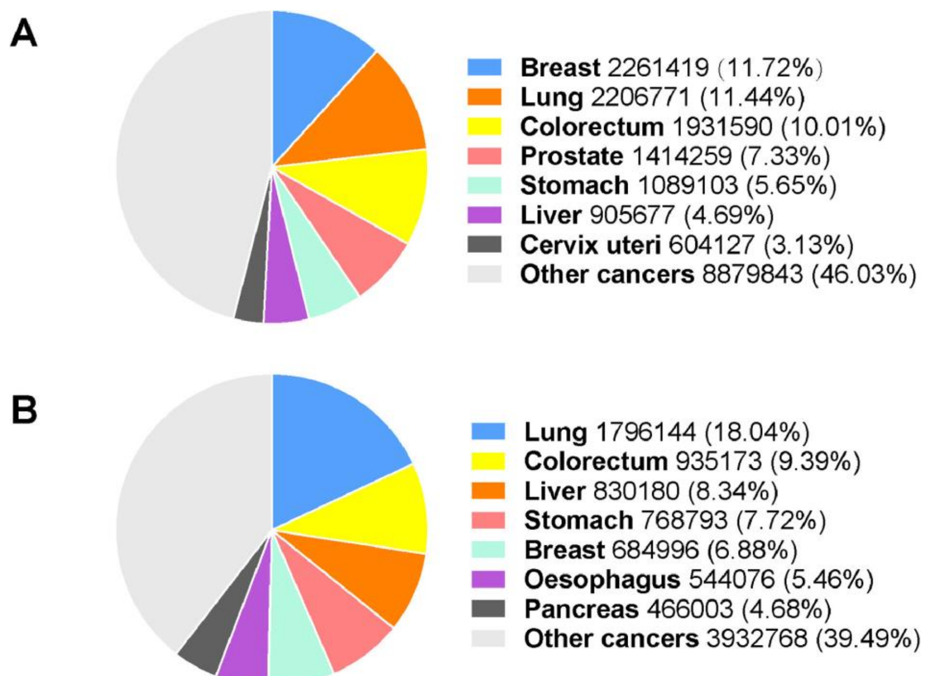

Figure 2.2: Estimates of the various cancer incidents and death rates worldwide, including breast cancer: (**A**) New cancer incidents estimated globally in 2020 with breast cancer included; (**B**) Global estimates of cancer mortality rates (Xi and Xu 2021).

2.1.3 Breast cancer aetiology

Several studies have suggested that the onset of breast cancer is attributed to ovarian hormones such as oestrogens and progestogens (Li et al. 2008; Iversen et al. 2011; Dall and Britt 2017). Moreover, obesity before the onset of menopause decreases the risk of breast cancer and obesity post-menopause increases the risk of breast cancer (Travis and Key 2003; Iversen et al. 2011; Kotsopoulos 2019; Mohanty and Mohanty 2021). This is caused by the adipose tissue that acts as an oestrogen biosynthesis reservoir following menopause (Mohanty and Mohanty 2021). The high serum oestrogen levels and the intensified peripheral oestrogen site of production have been considered reasons for breast cancer development in overweight women following menopause (Mohanty and Mohanty 2021). Additionally, it was shown that females with blood group A and Rhesus positive experience a high risk of breast

cancer development, whilst those with Rhesus negative and blood group AB had the lowest breast cancer risk (Momenimovahed and Salehiniya 2019).

It has also been shown that inherited gene mutations of BRCA1, BRCA2, and PALB-2 greatly elevate the risk of cancer (World Health Organization 2021a). Females found to display mutations in these major genes are advised to consider surgical removal of the breasts, albeit it should not be rushed because thorough decisions and procedures should be followed (World Health Organization 2021a). The early onset of breast cancer is common in females that harbour the mutations of BRCA1, with approximately 80% of them presenting with aggressive triple-negative tumours (Johnson et al. 2018). Additionally, 50% of adolescents and young adults who are diagnosed with cancer of the breast before the age of 30 present with mutations of BRCA1, BRCA2, and TP53 (Johnson et al. 2018). Moreover, the risk of developing breast cancer in adolescents and young adults is increased by the germline PALB-2 by at least eightfold (Johnson et al. 2018).

Exogenous hormones may also contribute to elevating the risk of breast cancer. These hormones may be found in oral contraceptives or through oestrogen replacement therapy (Vaz-Luis and Partridge 2018). The breast cancer risk is elevated in adolescents and young adults who use the above hormones, particularly teenagers who carry germline BRCA1 mutations (Johnson et al. 2018). However, a study by Balekouzou and colleagues illustrated that the ovulatory menstrual cycle may protect against developing breast cancer whilst breastfeeding decreases the risk of developing breast cancer in young adults as well as adolescents (Balekouzou et al. 2017; Johnson et al. 2018). Other risk factors include the ingestion of a high-fat diet during childhood and adolescence and the consumption of alcohol, which contributes to the development of breast cancer by elevating oestrogen production (Mattisson et al. 2004; Simbre et al. 2005; Hermawan et al. 2010; Sieri et al. 2014; Liu, Nguyen, and Colditz 2015; Mohanty and Mohanty 2021). It was also outlined that low body mass index, as well as substantial weight gain in adolescents and young adults, elevates the risk of oestrogen receptor-negative breast cancer, and in contrast, the risk is decreased by vigorous exercise (Johnson et al. 2018). Furthermore, studies have indicated that currently employed breast cancer treatments lead to various side effects: cardiomyopathy and developing chemo-resistance (Bird and Swain 2008; Trachtenberg et al. 2011a; Kalyanaraman 2020). In order to prevent the side effects of cancer treatments, the pathophysiology of breast cancer and the anti-cancer drugs' mechanisms must be fully understood.

2.1.4 Breast cancer pathophysiology

Breast cancer development is a result of damage to the deoxyribonucleic acid (DNA) and mutations to the genes that can be influenced by exposure to oestrogen receptors, progesterone receptors, as well as human epidermal growth factor receptor 2 (Alkabban and Ferguson 2022; Chalasani et al. 2021). The immune system attacks cells with abnormal DNA in cancer-free individuals. Failure to fight the rapid abnormal DNA growth leads to tumour growth. The reoccurrence of breast cancer is predicted based on tumour markers (Choi 2022). An example may include metastatic breast cancer occurring within a period of three years in patients who do not show any tumour markers or occurring ten years later following the first diagnosis and treatment in estrogen receptor-positive tumour patients (Choi 2022). Breast cancer can occur in two tissue types: the ductal and lobular epithelium (Nurses 2018). While most cancerous abnormalities of the breast originate from within the ductal epithelium, the cancerous cells may also arise within the lobular glands (Nurses 2018). In other cases, DNA defects or pre-cancerous genes such as BRCA1 and BRCA2 may be inherited (Alkabban and Ferguson 2022). Therefore, a family history of breast cancer is considered to elevate the risk of development of breast cancer (Alkabban and Ferguson 2022). Women need to be screened for breast cancer constantly, especially if they come from a family with a history of breast cancer (Loomans-Kropp and Umar 2019).

2.1.5 Breast cancer diagnosis

Breast cancer is diagnosed by screening a patient or conducting a diagnostic exam following an experience of a symptom (McDonald et al. 2016). Mammography has been proven to improve breast cancer detection as well as reduce mortality by 19%. It is recommended when a woman reaches the age of 45 (McDonald et al. 2016). Women who carry the mutations of BRCA1 and BRCA2 have a higher risk of developing breast cancer (Casaubon, Kashyap, and Regan 2021). Therefore, an annual mammogram is required for individuals with a family history of cancer at the earliest age of 25 but not later than 40 years. Additionally, the utilisation of magnetic resonance imaging (MRI) as an adjunct to mammography is suggested by the American Cancer Society for classifying and measuring the extent of breast cancer as well as the size of the tumour (McDonald et al. 2016).

2.1.6 Breast cancer classification

The TNM classification system is used to categorise breast cancer according to various criteria (Sparano 2021). The "T" in the classification system indicates the size of the tumour and how penetrated the tumour is. The total number of lymph nodes that have cancer is

indicated by the "N" in the classification system. The "M" represents distant metastasis, which shows if cancer has spread to other body parts. The reason for classifying is to choose the best treatment plan, and specific treatments are effective depending on the type of breast cancer. The TNM factors were grouped by the American Joint Committee on Cancer into overall stages of cancer (Sparano 2021).

2.1.7 Breast cancer stages and grades

The first stage (I) indicates that the cancer is still localised and has not spread (Dersarkissian 2021). Stage two (II) refers to the size of the cancer that has increased but has not spread. The third (III) stage indicates that the cancer cells may have spread to tissues or lymph nodes. In the fourth (IV) (final) stage, cancer has spread to other vital organs within the body (Dersarkissian 2021). The subtype and grade of breast cancer distribution vary with age, and the phenotypes are more aggressive in adolescents and young adults as compared to older women (Johnson et al. 2018). The Basal-like and HER-2 enriched tumours of the breast are the types of breast cancer commonly found in young women as compared to older ones (Johnson et al. 2018). In addition, pre-menopausal females presenting with invasive breast cancer have an increased risk of developing oestrogen receptor-negative, progesterone receptor-negative, as well as pathologic third-grade tumours (Johnson et al. 2018). However, for one to know the stage of cancer, the cancer grade must be specified (Verity 2021). This is a process of measuring how fast the growth of the cancer is and how it looks when comparing them to the normal cells (Verity 2021). This will provide better knowledge of the type of treatment that can be administered to the cancer patient.

2.2 Current cancer treatment therapies

Cancer is a disease in which cells grow uncontrollably (Reilly 2007). As discussed previously, the various clinical cancer stages are characterised by the type, size, and degree of metastasis, which forms the determining factor for the specific anti-cancer treatment strategy (Zhang et al. 2020). Various conventional treatment therapies can be used for breast cancer: chemotherapy, hormonal therapy, radiation, and surgery (Metri et al. 2013; Tang et al. 2016; Waks and Winer 2019). In addition, immunotherapy can also be included in one's treatment plan. Immunotherapy is when a patient utilises their own immune system to fight cancer (American Cancer Society 2019). This treatment therapy boosts and changes how the patient's immune system works to detect, locate and fight cancer cells (American Cancer Society 2019; Society 2022). Moreover, targeted therapy can also be used as a treatment for

cancer. Targeted therapy can be utilised as monotherapy or combined with other treatments, including radiation, chemotherapy, or surgery and can also be used as an adjuvant (Society 2022).

The most common surgical procedure employed for breast cancer is mastectomy, in which there is the removal of the entire breast. This includes all the breast tissue and, at other times, the surrounding tissues as well (American Cancer Society 2021b). Breast-conserving surgery (Lumpectomy) can also be performed where only part of the breast tissue that contains the cancer is removed (American Cancer Society 2021b). A patient who had a mastectomy does not need to undergo radiation therapy as compared to one who has performed a lumpectomy (American Cancer Society 2021b). Radiation therapy is the treatment that destroys cancer cells with high-energy particles that include X-rays, electron beams and protons.

Additionally, hormonal therapy is also used as a treatment for breast cancer. Oestrogen and progesterone hormones have been shown to affect some types of breast cancers as they help the tumours grow (American Cancer Society 2021b). However, hormonal therapy prevents the hormones from attaching to the protein receptors found in the breast cancer cells (American Cancer Society 2021b). Hormone therapy can be administered as an adjuvant following surgery to reduce the reoccurrence of breast cancer.

Chemotherapy can also be utilised to treat breast cancer. The anti-cancer drugs may be injected into a patient's vein or administered orally (American Cancer Society 2021a). Chemotherapy may be recommended following surgery (adjuvant chemotherapy) to kill the remaining microscopic cancer cells or prior to surgery (neoadjuvant chemotherapy) to shrink the tumour size for it to be removed with a less extensive surgery (American Cancer Society 2021a). If following neoadjuvant chemotherapy, cancer cells are still present, then more chemotherapy will be recommended (adjuvant chemotherapy) to reduce the recurrence of cancer (American Cancer Society 2021a).

Over the past decade, a significant improvement in cancer-patient outcomes has been indicated due to the advancement of anti-tumour antibiotics such as anthracyclines (ATCs), **Figure 2.2** (Hortobagyi 1997). These anti-tumour antibiotics change the DNA found in the cancer cells to prevent them from growing and multiplying (American Cancer Society 2019). According to Abdullah and colleagues, ATCs have become the cornerstone of chemotherapy for various cancers, especially since many ATCs are already effective and approved as anti-cancer drugs (Panche, Diwan, and Chandra 2016; Abdullah et al. 2019). They include

Daunorubicin, Epirubicin, Idarubicin and Doxorubicin (Panche, Diwan, and Chandra 2016; Marinello, Delcuratolo, and Capranico 2018; Abdullah et al. 2019).

Figure 2.3: Anthracyclines (ATCs): Various ATCs are used as chemotherapeutic agents to treat different malignancies. They include Doxorubicin, Epirubicin, Daunorubicin, and Idarubicin (Marinello, Delcuratolo, and Capranico 2018).

Of interest in this study is Doxorubicin (Dox), initially isolated in the 1950s from the soil bacterium *Streptomyces peucetius* (Borgatti 2013). Dox is also referred to as Adriamycin. The United States Food and Drug Administration (FDA) approved the chemotherapeutic drug Dox, which is frequently used to treat various cancers such as lung cancer, stomach, Hodgkin’s lymphoma, ovarian cancer, and breast cancer (McGowan et al. 2017b).

It has improved the cancer survival rate from 40% in 1971 to more than 70% in 2016 for both males and females (**Figure 2.4**) (Lipshultz et al. 2011; McGowan et al. 2017b; American Cancer Society 2022). However, it has been shown that administering Dox in high doses can permanently damage the heart of patients (American Cancer Society 2021a). As a result, cumulative doses are recommended when this drug is administered (American Cancer Society 2021a).

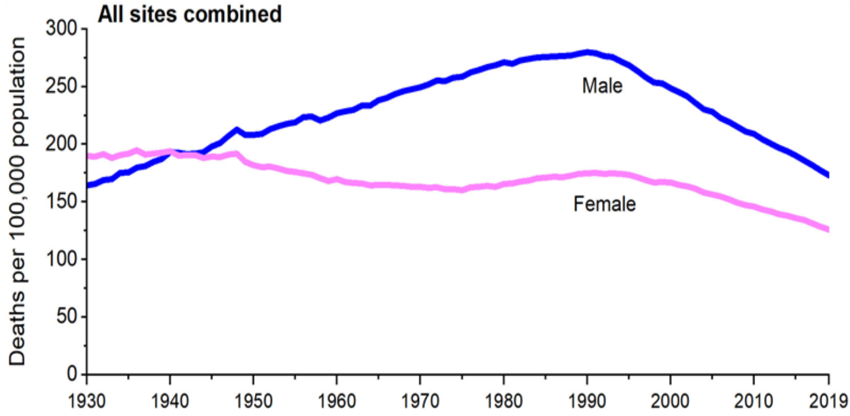

Figure 2.4: Improvement of cancer-patient outcomes: The number of cancer death cases has decreased globally over the years due to the administration of chemotherapeutic agents (ATCs) (Siegel et al. 2022).

Unfortunately, the use of Dox is associated with dose-dependent side effects (**Figure 2.5**) (Sangweni et al. 2020). This includes the development of oedema, which leads to heart failure, adverse effects on the liver, nerve damage, and elevating the risk of developing leukaemia as well as chemo-resistance (McGowan et al. 2017b). Breast cancer remains to be investigated as it has been shown to have several side effects associated with current chemotherapeutic regimens. However, the pathophysiology and the mechanism of action of breast cancer drugs require better understanding in order to circumvent common side effects and to reduce cancer cell proliferation.

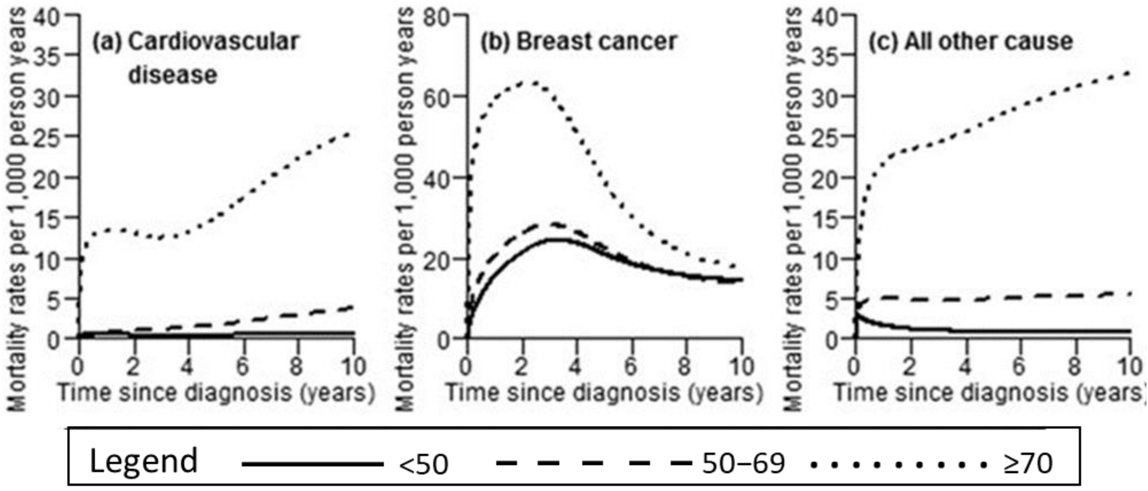

Figure 2.5: Administration of ATCs improves cancer patient outcomes at the expense of increasing cardiovascular deaths (Bekerman et al. 2014; Gernaat et al. 2017).

2.2.1 Mechanism of action of Dox

Doxorubicin's mechanism of action entails its ability to interject within the base pairs of DNA (Johnson-Arbor and Dubey 2022). This causes the DNA strands to break and inhibit the synthesis of both DNA and RNA (Johnson-Arbor and Dubey 2022). Dox causes the inhibition of the enzyme Topoisomerase II, which normal function is to untangle the supercoils and create space for new strands of DNA ("Topoisomerase: Definition & Function" 2017). However, in a cancer disease state, the Dox inhibition causes DNA damage (disrupting adenosine triphosphate (ATP) production), which leads to the induction of cell death (Octavia et al. 2012; Abd-Rabou et al. 2017; Sangweni et al. 2020; Johnson-Arbor and Dubey 2022). Furthermore, combining Dox with iron limits DNA synthesis by causing free-radical-mediated oxidative damage (Johnson-Arbor and Dubey 2022).

2.3 Role of oxidative stress in cancer

Oxidative stress is a cellular process that underpins a variance in producing reactive oxygen species (ROS) and endogenous antioxidants, potentially leading to cellular damage (Hecht et al. 2016; Pizzino et al. 2017). Cellular ROS are produced endogenously during mitochondrial oxidative phosphorylation and may also be generated during cellular interactions with xenobiotic compounds (Ray, Huang, and Tsuji 2012). Endogenous antioxidants are required to mitigate ROS-mediated injury (Fuchs-Tarlovsky 2013). Antioxidants such as glutathione (GSH) are compounds that scavenge free radicals (superoxide, hydrogen peroxide, and hydroxyl anion) by maintaining oxidation-reduction (redox) equilibrium (**Figure 2.6**) (Fang, Yang, and Wu 2002; Fuchs-Tarkovsky 2013; Simpson, Pase, and Stough 2015; Aggarwal and Tuli 2019). GSH comprises amino acids cysteine, glutamic acid, and glycine (**Figure 2.6**), which modulates the cellular redox status (Forman, Zhang, and Rinna 2009; Harvey et al. 2009; Simpson, Pase, and Stough 2015).

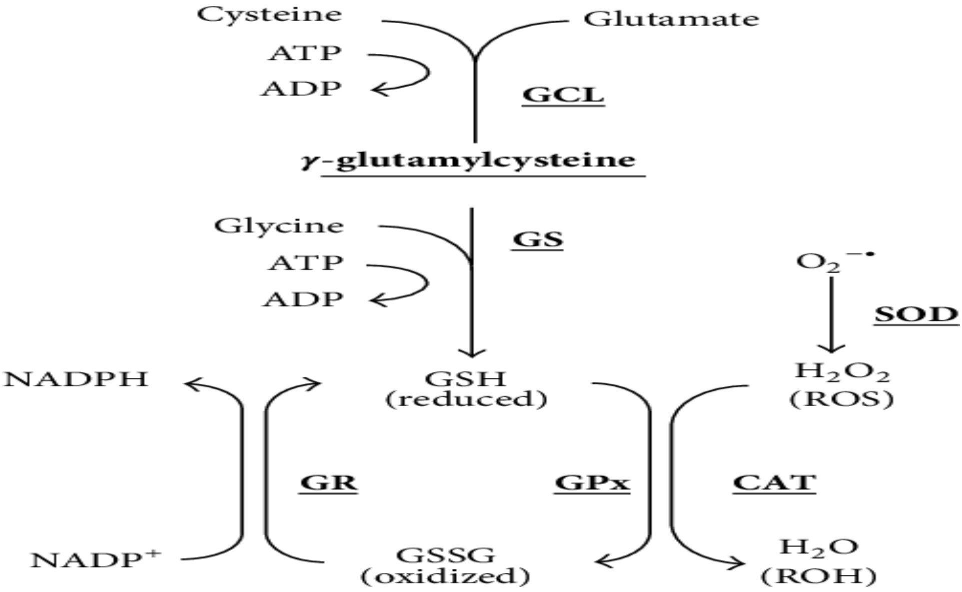

Figure 2.6: Glutathione reduction-oxidation reaction: Glutathione (GSH) consists of three amino acids. Glutathione peroxidase (GPx) is involved in the detoxification of ROS. The production of hydrogen peroxide (H_2O_2) is the result of the conversion of superoxide anion (O_2^-) by superoxide dismutase (SOD). An oxidised form, glutathione disulfide (GSSG), is produced and recycled back to GSH, utilising glutathione reductase enzymatic reaction. A cofactor, nicotinamide adenine dinucleotide phosphate (NADPH), is required for a redox reaction to occur (Simpson, Pase, and Stough 2015).

The decreased level of GSH, accompanied by an increase in ROS, may lead to the induction of oxidative stress, including the direct oxidative impairment of DNA, lipids, and proteins (**Figure 2.7**) (Gesellschaft and Mbh 2016; Lee et al. 2017). Lee and colleagues have hypothesised that DNA damage takes part in the initiation of carcinogenesis of the breast (Lee et al. 2017). In contrast, an increase in GSH levels has been shown to promote tumour growth by protecting breast cancer cells against cell death (Balendiran, Dabur, and Fraser 2004; Valenti et al. 2023). This reduces the effectiveness of treatment, contributing to the development of chemotherapeutic resistance (Balendiran, Dabur, and Fraser 2004; Valenti et al. 2023). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates cellular antioxidant expression, including GSH (Ma, 2013). Harvey and colleagues demonstrated that Nrf2 maintains the homeostasis of GSH by altering the *de novo* synthesis and modulating the redox state of GSH (Harvey et al. 2009). In addition, Nrf2 protects the cells from oxidative stress (Harvey et al. 2009). The imbalance between ROS and antioxidants leads to direct oxidative impairment (**Figure 2.7**) (Lee et al. 2017). Lee and

colleagues also suggested that ROS production and the resultant oxidative stress are the major aetiology leading to various cancers, including breast cancer development.

Elevated oxidative stress may also result in cytotoxicity, cause the inhibition of cell proliferation and cause apoptosis or necrosis. In contrast, a decline in oxidative stress may lead to DNA damage, mutation, and proliferation of cells and may eventually cause carcinogenesis (Isnaini et al. 2018).

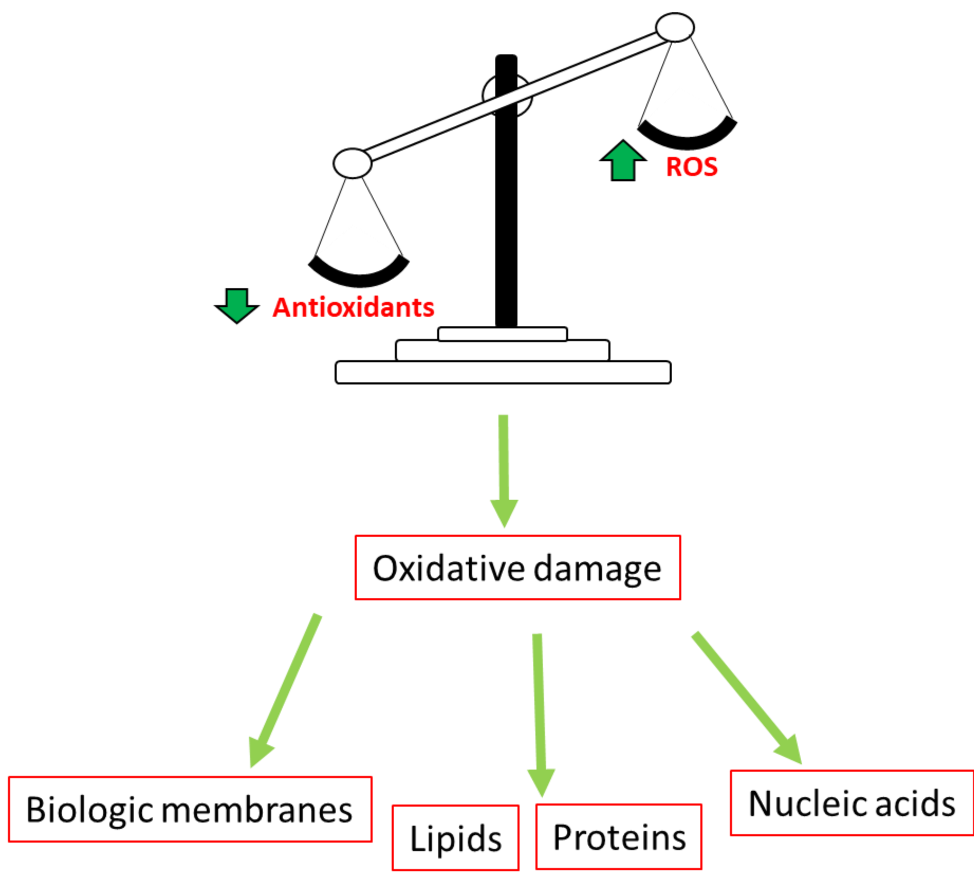

Figure 2.7: Mechanism of oxidative stress: Increased levels of ROS trigger activation of stress-related pathways. The imbalance between free radicals and antioxidant levels in the body leads to oxidative damage to cells. The oxidative damage causes protein lesions. This damage may lead to cancer, induction of apoptosis, heart failure, or damage to lipids, proteins and nucleic acid (Islam and Shekhar, 2015; Sultana et al. 2022). Figure created by Miss M Moremane using Microsoft Powerpoint 2013.

2.4 Antioxidants and breast cancer

Cells fail to maintain their own homeostasis because of the variance in oxidative stress (Griñan-lison et al. 2021). This leads to the induction of DNA damage and, therefore, cell death. Irreversible damage to the cells leads to the development of cancer. Thus, cancer development is caused when the pro-apoptotic pathways are activated (Griñan-lison et al. 2021). Increased ROS levels in cancer cells compared to normal cells are considered to be the cause of carcinogenesis as they sustain malignant phenotypes and promote the proliferation of cells as well as metastasis and angiogenesis (Hayes, Dinkova-Kostova, and Tew 2020). Cancer cells thrive on low levels of oxygen, and evidence has shown that oxidative stress and lipid peroxidation are associated with the development of breast cancer (Kang 2002; Perillo et al. 2020). Natural products such as flavonoids and phenolics are effective in cancer as they suppress carcinogenesis (both early and late stage) (Slika et al. 2022). Studies have shown that antioxidants may cause apoptosis in tumour cells but also inhibit the proliferation of cancer cells and spare normal cells (Sigounas et al. 1997; Prakash et al. 2002; Borek 2004; Zeisel 2004). Tumour cell hyperproliferation results from the increased production of ROS (Hayes, Dinkova-Kostova, and Tew 2020). The cancer cells accomplish this by raising their oxidant status for ROS-driven proliferation optimisation, concurrently avoiding the threshold of ROS that would lead to apoptosis and ferroptosis (Hayes, Dinkova-Kostova, and Tew 2020). Reports have been made that mitochondrial ROS leads to the promotion of cell death.

Meanwhile, NOX-generated ROS promotes the proliferation and migration of cells (Perillo et al. 2020). Xue and colleagues demonstrated that ROS regulates the chemoresistance and chemosensitivity of cancer to various tumour drugs (Xue et al. 2020). Additionally, Nrf2 plays the same role as ROS, resulting in chemoresistance of cancer drugs (Xue et al. 2020).

2.5 Nrf2 and cancer

The transcription factor Nrf2 is considered a cellular protector (for both normal and cancerous cells) that stimulates how genes express themselves (Zimta et al. 2019). It depicts a double role of leading to the inhibition of cancer development and also promotes the progression of cancer as well as resistance to chemotherapy (Xue et al. 2020). It has sequences of antioxidant response elements in the promoter region (Zimta et al. 2019). The hyperactivation of Nrf2 has been shown to induce proliferation as well as the overgrowth of tumour cells. This prevents tumorous cells from going through apoptosis, causing the cells to be resistant to both chemotherapy and radiotherapy (Zimta et al. 2019). The Nrf2 gene has

been demonstrated to be stabilised by K-RAS and c-MYC oncogenes, which induce the production of intracellular ROS (Perillo et al. 2020). Moreover, it has been shown that mutant p53, which contributes to the proliferation of cancer and metastasis, causes the activation of Nrf2 gene transcription (Lisek et al. 2018; Hu et al. 2021). It has also been reported that targets of the gene Nrf2, including heme oxygenase 1 (HMOX1), initiate the development of cancer as they prevent the oxidative stress effect in cells that are transformed (Diehn et al. 2009; Lo and Matthews 2013; Perillo et al. 2020). However, the HMOX1 target gene is repressed by p53 (Lisek et al. 2018).

2.6 Role of tumour protein p53 in cancer

Apoptosis is known as a vital defence mechanism for different processes, such as normal cell turnover and how the immune system functions (Elmore 2007). Different stimuli and conditions can trigger it; not all cells can succumb to the same stimuli (Elmore 2007). Chemotherapeutic agents induce apoptosis through the p53-dependent pathway (Elmore 2007). Therefore, apoptosis is dependent on the therapeutic efficiency of anti-cancer drugs in targeting cancer cells. Since the nuclear transcription factor, p53 regulates the fate of the cell in response to damage to the DNA, it is required to have a therapeutic approach that activates the p53-mediated pro-apoptotic pathway. It has a principal role in controlling antioxidant gene expression (Perillo et al. 2020). Moderate levels of ROS lead to the inhibition of p53, whilst high levels encourage the promotion of its expression (Perillo et al. 2020). The p53 tumour suppressor (TP53) also regulates important processes such as cell metabolism, progression of cancer cells, as well as senescence in response to different stresses on the cell (Fedorova et al. 2020). The p53 gene has been shown to mutate in various tumours, including breast cancer (Duffy, Synnott, and Crown 2018; Fedorova et al. 2020). In some cases, these p53 mutations may show chemo-resistant phenotypes (Ozaki and Nakagawara 2011). In cancer cells, there is downregulation of pro-apoptotic proteins such as Bax and the overexpression of the anti-apoptotic protein, Bcl-2. This may result in cancer cell survival. Moreover, the TP53 function may be dysregulated by several cancer cells that evade apoptosis. The induction of cell cycle arrest or apoptosis depends on how extensive DNA damage is (Ozaki and Nakagawara 2011). The cell cycle arrest mediated by the p53 gene permits the cells that have their DNA damaged to be repaired. However, when there is DNA damage to the cells, p53 exercises its pro-apoptotic function to remove cells with extreme DNA damage and prevent the transferral of impaired DNA to daughter cells. Therefore, p53

is well known for its ability to maintain the integrity of genomes (Ozaki and Nakagawara 2011).

2.7 Apoptosis (programmed cell death) on cancer development

Apoptosis, programmed cell death, is a self-destructive process of cells (Sreelatha, Jeyachitra, and Padma 2011). The apoptotic pathway is activated through increased ROS production and DNA damage (Pfeffer and Singh 2018a). Two signalling pathways activate cellular death, namely the intracellular (mitochondrial) and extracellular (death receptor) apoptotic pathways (Pfeffer and Singh 2018a; Sangweni et al. 2020). The intracellular signals are characterised by DNA damage, whilst the extracellular signals have cytotoxic T cells that produce death-inducing signals (Jan and Chaudhry 2019). In cancer, there is an inhibition of apoptotic pathways (**Figure 2.8**) through the up-regulation of anti-apoptotic proteins (Bcl-2) and the downregulation of the pro-apoptotic proteins (Bax), resulting in intrinsic resistance to chemotherapy (Pfeffer and Singh 2018a). Mitochondrial depolarisation is one of the main intrinsic mechanisms by which apoptosis is induced. In a study by Shabalala and colleagues, Dox activated p53, a tumour suppressor protein. The activation of p53 increased the expression of the Bcl-2-like protein 4 / B-cell lymphoma 2 (Bax/Bcl-2) ratio. This led to increased mitochondrial membrane permeability, releasing cytochrome c and aiding the activation of apoptosis through elevated caspase-3 expression (**Figure 2.8**) (Shabalala et al. 2017). Dox has also been shown to inhibit topoisomerase II, an enzyme required by cancerous cells that promotes division and proliferation by intercalating with cellular DNA (Mitry and Edwards 2016). Additionally, Dox has also been demonstrated to induce apoptosis by activating the enzyme Poly (ADP-ribose) polymerase-1 (PARP-1) (Gungor-Ordueri et al. 2019).

The PARP-1 protein regulates developmental processes such as DNA damage signalling (Bouchard, Rouleau, and Poirier 2003; Thomas et al. 2019). It is also responsible for the proliferation as well as differentiation of cells. The PARP-1 protein detects and repairs DNA damage (Pascal 2018; Xie et al. 2020). Moreover, it has been shown that the activation of the PARP-1 protein may play a role in inducing side effects like Dox-induced cardiotoxicity (Pacher et al. 2002). However, there is a need for alternative treatments, such as herbal compounds that have the potential to activate apoptosis and elicit minimal side effects in various cancers (Luo et al. 2019).

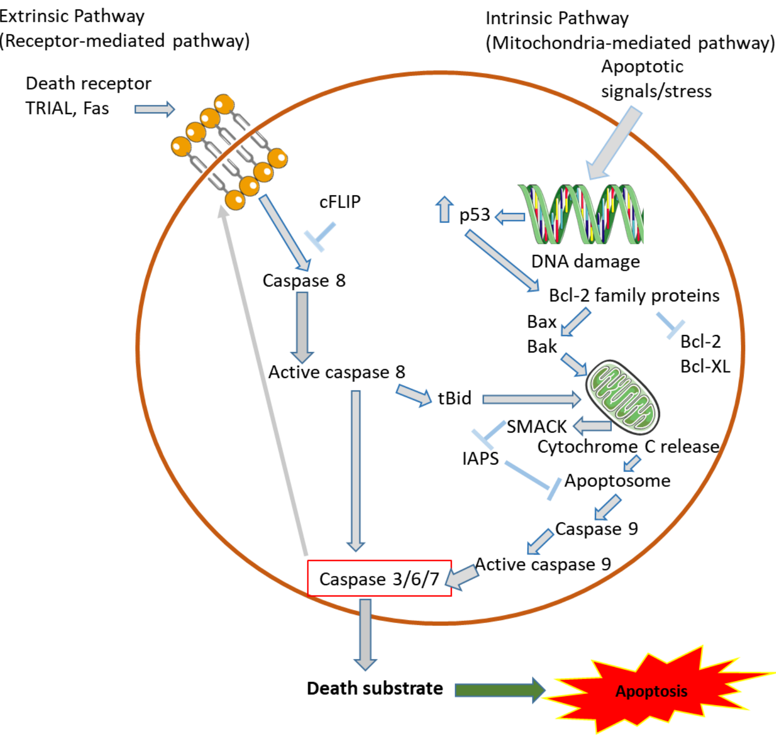

Figure 2.8: The intrinsic and extrinsic mechanism of apoptosis: Increased oxidative stress in cells or the activation of extracellular ligands induces DNA damage and mitochondrial impairment. The release of cytochrome c from the mitochondria forming an apoptosome with APAF-1, procaspase-9, and ATP initiates the caspase cascade. Adapted from Jan and Chaudhry (2019) and Irfan et al. (2022). Figure created by Miss M Moremane using Microsoft Powerpoint 2013.

2.8 The potential of herbal drugs in chemotherapeutic regimens

Over the years, the interest in exploring alternative treatment modalities has escalated (HemaIswarya and Doble 2006). People have utilised herbal medicines to overcome and prevent certain diseases, particularly in patients with medical conditions such as type 2 diabetes mellitus, breast cancer, and human immunodeficiency virus (Kassler, Blanc, and Greenblatt 1991; Burstein, Gelber, Guadagnoli, and Weeks 1999; Inamdar et al. 2010). Many traditional medicines have been investigated. Their antiproliferative effects were seen in different cancer cells (e.g., Pinocembrin, an herbal drug that has demonstrated anti-cancer properties by increasing apoptosis and necrosis in breast cancer cells) (Sangweni et al. 2020). *Sutherlandia frutescens*, also known as cancer bush, is often utilised by traditional healers in

SA to treat various ailments, including breast cancer (Gericke et al. 2001; Phulukdaree, Moodley, and Chuturgoon 2010).

Plant-based therapy, such as the use of flavonoids, has been extensively investigated for its protective properties against toxicities inferred by chemotherapy (Panche, Diwan, and Chandra 2016). Flavonoids are known polyphenolic compounds with antioxidant, anti-inflammatory, anti-carcinogenic, and cardio-protective properties (Panche, Diwan, and Chandra 2016). The use of plants for their therapeutic potential against various ailments has significantly increased over the years. Recently, natural products have contributed to the production of medicinal agents that treat chronic and acute human diseases (Eddouks et al. 2014). Studies have shown that plant-based medicines possess several biological activities, including anti-cancer properties (Inamdar et al. 2010; Jung 2014; Sangweni et al. 2020). However, the biological activities regarding the cancer treatment potential of several traditionally used plants are yet to be explored. Therefore, using a plant such as *Moringa oleifera* (MO) in traditional medicine in SA requires extensive investigation for its biological properties to be fully elucidated.

2.8.1 *Moringa oleifera*

Moringa oleifera (MO), from the family Moringaceae, is a tree native to India (**Figure 2.9**) and is found throughout SA (Mahmood, Mugal, and Haq 2010; Gopalakrishnan, Doriya, and Kumar 2016). It is commonly known as horseradish or the drumstick tree (Amaglo et al. 2010; Gopalakrishnan, Doriya, and Kumar 2016; Karim et al. 2018). MO is considered to be the “powerhouse” of essential nutrients. The leaves are rich in calcium, copper, magnesium, potassium, zinc, and iron. It contains vitamins A, B, C, D, and E (Gopalakrishnan, Doriya, and Kumar 2016).

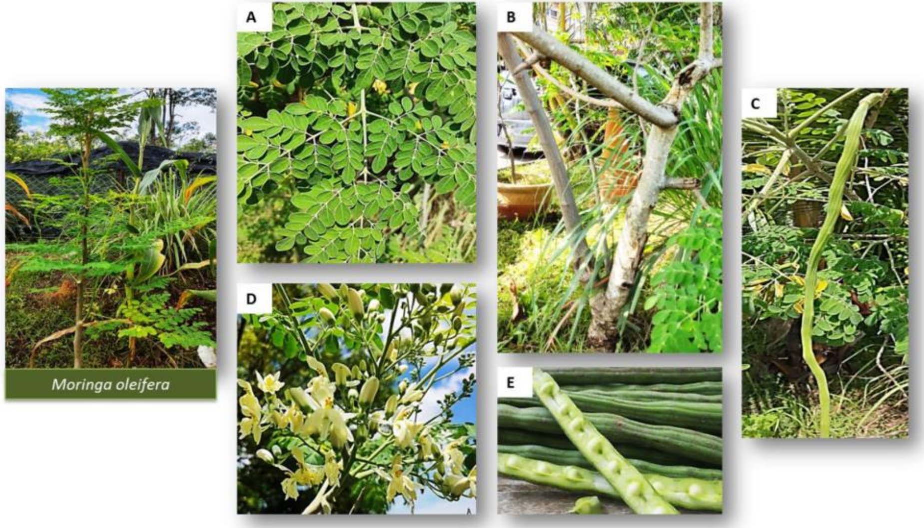

Figure 2.9: The various parts of *Moringa oleifera* tree: (**A**) Leaves, (**B**) Stem and Bark, (**C**) Pods, (**D**) Flowers and sepals as well as (**E**) seeds (Karim et al. 2016).

MO has been traditionally used to treat viral and bacterial infections, inflammation, and hyperglycemia (Zahirah et al. 2018). It has been used traditionally in many countries. For example, Nigerians use MO to treat fertility disorders in females and increase the male fertility capacity (Fidrianny, Kanapa, and Singgih 2021). The seeds of MO were also proven to purify water in African countries such as Ethiopia (Delelegn, Sahile, and Husen 2018). MO contains Niazimicin, a thiocarbamate that suppresses cancer cell proliferation (**Figure 2.10**) (Miyoshi et al. 2004; Tiloke et al. 2018). It also contains benzyl isothiocyanate, a compound that increases intracellular ROS and apoptosis, emphasising the anti-cancer potential of MO (Lee and Shacter 1999; Nakamura et al. 2002; Miyoshi et al. 2004; Hermawan et al. 2012; Tumer et al. 2015).

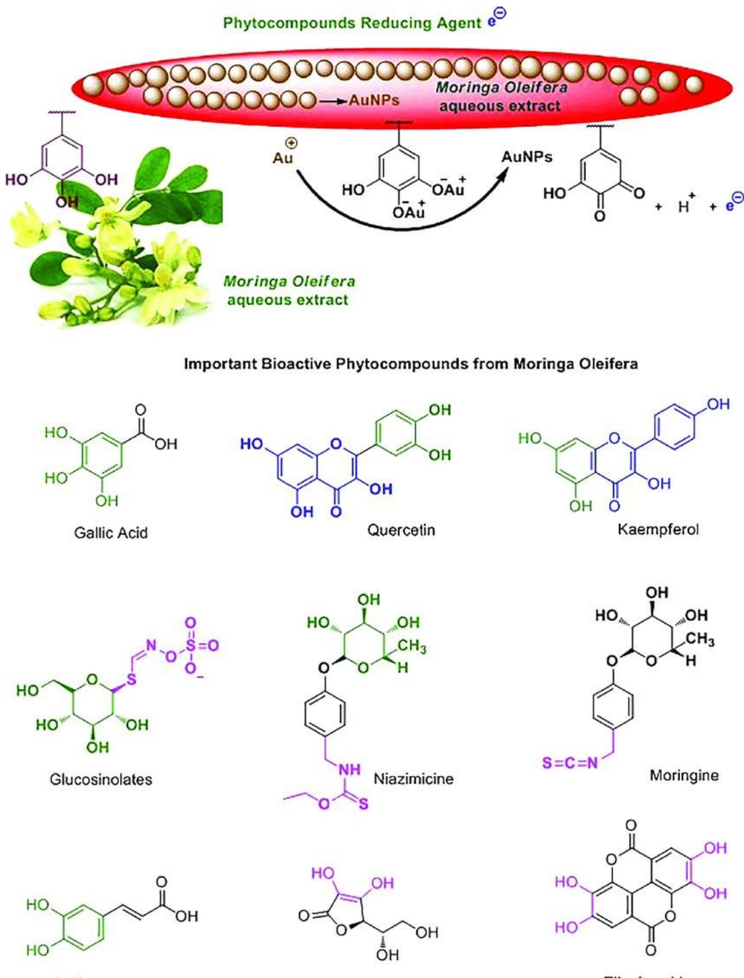

Figure 2.10: Chemical structures of bioactive compounds of MO. There are various compounds found in MO that make it a good chemotherapeutic agent. This includes Niazimicin as well as Kaemferol and Quercetin (Tiloke et al. 2018).

MO has an abundance of phytochemicals present in the leaves, pods, and seeds, including sterols, flavonoids, and anti-cancer agents such as glucosinolates, isothiocyanates, and glycosides (Table 2.1) (Gopalakrishnan, Doriya, and Kumar 2016). MO also contains Zeatin, which is an anti-ageing compound. Zeatin has anti-cancer properties and is a good antioxidant (Islam et al. 2021). It also has bioactive compounds such as saponins and tannins that have anti-cancer properties and alkaloids (Table 2.1), which have the potential to be a cardiac stimulant (Vergara-Jimenez, Almatrafi, and Fernandez 2017; Islam et al. 2021). This means it can prevent cardiac problems induced by Dox (Islam et al. 2021).

Table 2.1: The phytochemicals present in MO. The leaves of MO have more flavonoids and Saponins present as compared to other compounds (Packialakshmi and Archana 2014).

Constituents	**Aqueous extract**
Alkaloids	++
Flavanoids	+++
Saponins	+++
Carbohydrates	++
Tannins	+
Steroids	++
Glycosides	+
Gums and mucilage	++
Lignin	ND
Phenols	+
Fixed oils and fats	++
Amino acids and proteins	++

***Note**: + = Low relative presence of compound; ++ = Moderate abundance of compound; +++ = Relative abundance of compound; ND = Not detected

In addition, studies showed that MO's high antioxidants and bioactive compounds play an important role in preventing heart damage inferred by chemotherapeutic agents such as Dox (Stohs and Hartman 2015). According to Gopalakrishnan et al. (2016), MO can also be used to treat malnutrition in children. It is described as the “miracle tree” for its fast-growing, multi-purpose, drought tolerance, and nutritional and medicinal properties (Gopalakrishnan, Doriya, and Kumar 2016). However, further research on MO and Dox herb-drug interaction is required as they influence cytochrome P450 enzymes (e.g., CYP3A4, CYP1A2, and CYP2D6 in liver cells) (Zordoky and El-Kadi 2008; Fantoukh et al. 2019; Zunica et al. 2021). Asare and colleagues also indicated that supplementing the leaf extract of MO can become toxic if the levels are higher than 3000 mg/kg body weight, but are considered safer if below 1000 mg/kg (Asare et al. 2012).

2.8.2. Anti-cancer properties of *Moringa oleifera*

Studies have demonstrated that MO can be used as an anti-cancer agent at established concentrations (Table 2). Both solvent and soluble leaf extracts of MO have been demonstrated to have effective anti-cancer properties (Gopalakrishnan, Doriya, and Kumar 2016). Furthermore, studies have indicated that the antiproliferative effect of MO may be attributed to its ability to elevate oxidative stress, leading to DNA fragmentation and induction of apoptosis in A549 lung cancer cells (Tiloke, Phulukdaree, and Chuturgoon 2013; Jung 2014; Abd-Rabou et al. 2017). Luo and colleagues found that phytochemicals in MO induced apoptosis by activating p53 in ovarian cancer cells (Luo et al. 2011). In addition, MO induced apoptosis by causing DNA damage in hepatocellular carcinoma cells ($HepG_2$) (Tiloke et al. 2019). MO leaves are regarded as good free radical scavengers because they contain quercetin-3-0 glucoside and kaempferol-3-0 glucoside (**Figure 9**) (Fidrianny, Kanapa, and Singgih 2021).

Table 2.2: ***Moringa oleifera*****'s anti-cancer effects**

Experimental model	**MO dose**	**Experimental outcome**	**Proposed mechanism**	**References**
Human B-lymphocyte plasmacytoma (U266B1 cell line)	Methanol extract IC_{50}: 0.32 µg/ml	Increased cytotoxic activity	Inhibition of cell proliferation	Parvathy and Umamaheshwari (2007)
Lung cancer (A549 cell line)	Soluble cold distilled water extract 200 µg/ml	Demonstrated anti-cancer activity by reducing the expressions of AKT, NFKB, ERK, and cyclin D1	Induced apoptosis by activating caspases	Jung (2014)
Lung cancer (A549 cell line)	Water-soluble extract IC_{50}: 166.7µg/ml	Reduced levels of GSH, induction of DNA damage as a	Apoptosis induced by activation of	Tiloke, Phulukdaree, and Chuturgoon

		result of decreased levels of PARP-1 and Nrf2	caspases	(2013)
Colorectal cancer (CRC) cell lines T84, HCT-15, SW480 and HT-29	Ethanolic seed extract IC_{50}: 0.001μg/ml	Caspases 9, 8, and 3 overexpression and elevated the production of ROS	Induced apoptosis by autophagy	Fuel et al. (2021)
Pancreatic cancer (Panc-1 cell line)	Aqueous leaf extract 0.75 mg/ml	Reduced the p65 expression	Inhibition of cell proliferation	Berkovich et al. (2013)
Breast adenocarcinoma (MCF-7) and epithelial breast cancer cell line (MDA-MB-231)	Crude methanolic leaf extract 50 and 25 μg/ml	Decreased cell growth	Apoptosis induced in a time- and dose-dependent manner	Ghosh (2013)
Cervical cancer (HeLa cell line)	Methanol leaf extracts IC_{50}: 70 μg/ml	A decrease in cell viability with increased apoptosis	Apoptosis induced by DNA fragmentation	Varalakshmi and Nair (2011)
Hepatocarcinoma (HepG$_2$) and breast adenocarcinoma (MCF-7)	Dichloromethane extract 100 μg/ml	Demonstrated anti-cancer activity by reducing mitochondrial membrane potential, cell viability, and increasing DNA damage	Induced apoptosis by up-regulating Bax proteins	Abd-Rabou et al. (2017)

Hep2 human epidermoid	Methanol extract 200 µg/ml	Induced DNA fragmentation	Induced apoptosis by elevating ROS	Krishnamurthy et al. (2015)

MO has also been demonstrated to increase the levels of antioxidants, leading to decreased oxidative stress (Albrahim and Binobead 2018). According to Suphachai (2014), both methanol and dichloromethane extracts of MO can suppress the growth of human colorectal adenocarcinoma cells (Caco-2) as well as breast adenocarcinoma (MCF-7) cells (Suphachai 2014). This was further supported by Karim and colleagues as well as Wang and colleagues, who found a reduced nuclear factor kappa B (NFKB) expression in MCF-7 breast cancer cells using the dichloromethane extract, which subsequently caused a decline in ROS formation (Ashikin et al. 2016; Wang et al. 2018; Liu et al. 2019). Although the potential of MO has been scientifically proven to possess an anti-cancer, antioxidant, and anti-inflammatory effect on various other cancer cell types, future studies that include toxicity levels in *in-vivo* studies and clinical trials should be considered. However, this current study investigated the anti-cancer potential of the aqueous leaf extract of MO, exploring its antioxidant and apoptotic effect on MCF-7 breast cancer cells. Moreover, additional studies are required to indicate if it is safe and effective in humans.

2.9 Conclusion

Based on the current literature, MO can be used for multiple purposes, such as purifying water and consumed as a source of various nutrients. In addition, MO can treat various ailments and has also been seen to have antiproliferative potential in various cancer cell lines. Most of the biological activities aided by MO are caused by the high flavonoid, glucosides, as well as the glucosinolates it contains. However, there is still a lack of information on the antiproliferative mechanism of MO aqueous leaf extract in breast cancer. Therefore, as there are investigations done previously on the pathophysiology of breast cancer and MO, more research can be conducted in addition to supplement the present literature.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Introduction

This chapter provides a detailed description of the methodology and study design utilised in this research study.

3.2 Research design

Oestrogen receptor-positive breast cancer cells (MCF-7) were used to investigate the antiproliferative potential of MO aqueous leaf extract. This study utilised an *in vitro* model. MCF-7 cells were seeded in culture until they reached an 80-90% confluency. The Methyl thiazoltetrazolium (MTT) assay determined the cellular metabolic activity of MCF-7 cells. Subsequently, experiments to determine antioxidant and ROS levels, as well as apoptosis, were conducted (Figure 3.1). Western blotting was performed to detect the protein expression of Bax, Bcl-2, Nrf2, p53, and PARP-1. The expression of mRNA was also quantified using qPCR.

IN VITRO STUDY

MCF-7 cells

Cultured in Complete culture media For 2-3 days (until reaching 80-90 sub-confluence)

Seeding, Treat, Treat, Treat, Experiment

0, 24, 48, 72 hours

Treatment Groups (*n=3*)
1. Control
2. Doxorubicin (Dox)
3. *Moringa oleifera* (MO)
4. MO + Dox

Biochemical Analysis

- MTT assay
- GSH assay
- DCFH-DA assay
- Caspase activity assay
- ATP assay
- Western blotting
- qPCR assay

Figure 3.1: Experimental design: MCF-7 cells were cultured in complete culture media for two to three days until they reached a sub-confluence of 80-90%. Cells were treated for 24, 48, and 72 hours for the methyl thiazoltetrazolium (MTT) assay. Thereafter, treatment groups included the untreated control group, Dox (positive control), MO (assess antimetastatic potential), and a combination of Dox and MO (assess potential synergistic effect).

Biochemical analysis was conducted: Methyl thiazoltetrazolium (MTT) assay, Glutathione (GSH) assay, Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay, and caspase activity were conducted. In addition, an Adenosine triphosphate (ATP) assay, western blotting procedure, and qPCR (quantitative polymerase reaction) for mRNA expression were also performed (adapted by Moremane, 2021).

3.3 Procedures and techniques

3.3.1 Culturing and maintenance of MCF-7 breast cancer cells

MCF-7 cells are oestrogen receptor-positive human metastatic breast cancer cells (Sangweni et al. 2020). These cells are utilised in *in vitro* models as they present several breast cancer-type characteristics. This includes the processing of oestrogen in the form of estradiol through the oestrogen receptors of the cell cytoplasm (Camarillo et al. 2014). The cells were originally purchased from the American type culture collection (ATCC) (catalogue number: HTB-22) and housed at the Department of Pharmacology, University of the Free State (UFS). Professor Mamello Sekhoacha donated frozen cells. Quality assurance was conducted as described below. Culturing of the cells (**Figure 3.2**) was conducted in complete culture media (CCM) comprising Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS). The supplement also contained 1% of penicillin and streptomycin, 1% L-Glutamine, and was maintained at tissue culture conditions (37ºC, 95% relative humidity, and 5% CO_2). The T25 sterile flasks were used as a culture dish, and 96-well flat-bottom plates for the subsequent experiments. The medium was changed every second day as required. The reagents for cell culture were obtained from Sigma Aldrich (SA).

Figure 3.2: Culturing and maintenance of the MCF-7 breast cancer cells (Thermofisher Scientific 2022).

3.3.2 The biosafety precautions for culturing MCF-7 breast cancer cell-line

Biosafety level 1 is fundamental for promoting good microbiological techniques (OMS 2004; Shrivastava 2017). Biosafety level 1 regulations were always adhered to inside the tissue culture laboratory. Personal protective equipment (PPE) was used during all laboratory procedures. These included a laboratory coat, closed shoes, shoe covers, face masks, hair nets, and gloves (OMS 2004; Shrivastava 2017). The gloves were aseptically removed after disinfecting with 70% ethanol. Following that, hands were washed with soap following the experimental procedures. The PPE worn inside the laboratory was designated for the tissue culture laboratory only and was prohibited from being removed from the laboratory and worn outside the laboratory.

When conducting experiments, the researcher employed aseptic techniques. All tissue culture procedures were conducted inside a sterile Airtech laminar flow hood (Biosafety level 2). The laminar flow creates a sterile working environment through the constant and steady flow of air using a high-efficiency particulate air (HEPA) filter that captures all particles from entering the biosafety cabinet (Sabherwal et al. 2020). A sterile work environment was maintained by sterilising all working surfaces, including consumables and reagents, with 70% ethanol. A cell culture incubator, temperature, humidity, and CO_2 controlled, was used to maintain the cell cultures. The incubator has an automated built-in decontamination cycle, which was routinely conducted.

In order to maintain a sterile environment and for equipment sterilisation and decontamination purposes, a method that is preferred is autoclaving. It is a procedure utilised in our experimental methodologies to kill harmful bacteria on our laboratory equipment using high-pressure steam (OMS 2004; Shrivastava 2017). All discarded waste materials were separated according to their type and then disposed of as recommended. Autoclaved distilled water was added inside the incubator to maintain humidity levels. The non-biological waste was disposed of in the household bins, whilst the contaminated biological waste (e.g., used broken glass slides and serological pipettes) was disposed of in the puncture-proof containers fitted with covers using a red colour-coded bag, and the sharp materials were disposed into yellow sharp bins (OMS 2004; Shrivastava 2017). The containers were labelled and, therefore, placed at a central point. The culture medium was disinfected with a hypochlorite (bleach) solution before being autoclaved and disposed of. All biohazardous materials were collected and discarded by the Compass Medical Waste services after being autoclaved as they navigate healthcare risk waste management effectively.

Quality assurance was conducted upon receipt of cell cultures from the Pharmacology department. The cells were tested according to cell culture standards for the presence of mycoplasma and fungi. The DNA staining method with Hoechst stain (Appendix H) was utilised for testing for mycoplasma (Sigma-Aldrich 2007; Jean 2017). The cells were cultured in complete culture media comprising a penicillin-streptomycin combination, which aided in preventing bacterial contamination. In case of contamination development during culturing, a hypochlorite (bleach) disinfectant was used to neutralise infection, followed by discarding the infected cell cultures. All decontamination incidents were recorded.

3.3.3 Extraction of *Moringa oleifera* leaf

MO leaves were obtained from the Durban region, KwaZulu-Natal, and verified by the KwaZulu-Natal herbarium (Accession number: 151125) (Appendix B). A total of 10g of air-dried leaves were crushed with a pestle and mortar. Then, 100 ml of de-ionised water was mixed into the crushed leave sample. The extract was boiled and stirred for 20 minutes to release nutrients to the solvent (deionised water) and remove residual substances that may have been there during harvesting. Subsequently, the extract was transferred to 50 ml conical tubes and centrifuged for 10 minutes at room temperature. The MO upper aqueous layer was removed and stored at 4℃ after being lyophilised. The extract stock solution was prepared by

dissolving 5mg of MO in 1 ml of CCM. The solution was filter-sterilised with a 0,22 µm filter (Tiloke, Phulukdaree, and Chuturgoon 2013).

3.3.4 Preparation of Doxorubicin hydrochloride

Dox was prepared by dissolving 2 mg of Dox powder (MW 579.98 g/mol) in 1 ml dimethyl sulfoxide (DMSO). A starting stock concentration of 1000 µM was prepared and serially diluted to prepare the working concentrations and log10 dosage range of 0.01, 0.1, 1, and 10 µM. An IC_{50}, the concentration of Dox that caused a defined effect on 50% of a given population of the MCF-7 breast carcinoma cells, was determined.

3.3.5 Cell viability assay

A methyl thiazoltetrazolium (MTT) assay was conducted to determine the cytotoxicity of MO and Dox on MCF-7 cells. The assay measures cell viability through metabolic activity (Figure 3.3). The number of viable cells corresponds with the colour intensity shown on the photometric measurements after formazan has been dissolved in DMSO (Abd-Rabou et al. 2016). Cells were incubated with different MO concentrations (0 – 5000 µg/ml) and Dox concentrations (0.01 - 10 µM) in three replicates, followed by incubation for a period of 24, 48, and 72 hours. The untreated control cells were incubated with CCM only. Following the treatment period, the supernatant was removed, and cells were washed with 100 µL PBS. PBS was removed, and the cells were exposed to 20 µL MTT salt solution (5mg/ml) with 100 µL CCM. Cells were incubated for three hours. The supernatant was removed, and 100 µL of DMSO was added. The cells were then incubated for 30 minutes. The optical density of the formazan product was measured at 570 nm and 690 nm wavelengths utilising a spectrophotometer (Glomax® Discover Microplate Reader). The cell viability percentage was determined, and Graph Pad Prism V 9.4.0 software was used to plot the dose-response curve using non-linear regression analysis relative to the control. The IC_{50} concentration determined was used in all subsequent assays.

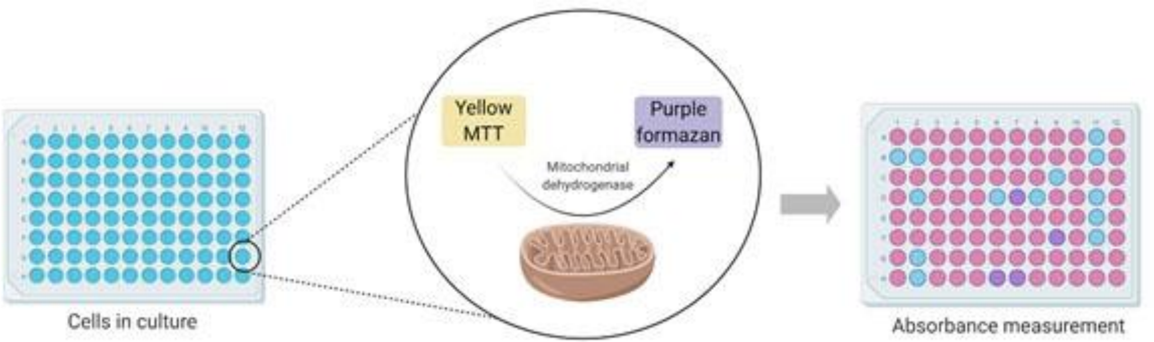

Figure 3.3: Visual representation of the MTT assay (Axion Biosystems 2022).

4. Treatment protocol

The most effective dose was acquired for 72 hours, and the MCF-7 breast cancer cells were treated with MO individually to investigate its antiproliferative effect. Moreover, cells treated with Dox only were used as the positive control treatment group. Furthermore, cells were co-treated with the pre-determined IC_{50} of Dox and MO to assess the anti-cancer interaction between Dox and MO when used at an equimolar dose ratio 1:1 (Figure 3.1). Untreated cells served as the control.

5. Oxidative stress determination

5.1 Assessment of glutathione using luminometry

Oxidative stress causes injury to the cells, resulting in DNA damage. Glutathione is important in protecting cells against oxidative stress (Forman et al. 2009). The levels of GSH were detected and quantified using the GSH-Glo^{TM} assay (Promega, Madison, WI, USA), as outlined by Tiloke et al. (2013). This assay converts the luciferin derivative into luciferin when GSH is present and is therefore catalysed using glutathione S-transferase (Figure 3.4). Following treatment for 72 hours, cells were trypsinised and centrifuged for 10 minutes at 2000 rpm (720 x g) (Hermle Labortechnik GmbH, Z 32 HK). A cell count was conducted, and cells were then resuspended in 1 ml PBS for each treatment group. A 96-well plate was used, and 50 µl of standards (50, 25, 12.5, 6.25, 3.125, 0 µM) were added in duplicate. Following that, 50 µl of MCF-7 cells (20 000) in PBS were added into each well. A volume of 50 µl of the GSH-Glo^{TM} working solution reagent (Reaction Buffer- 48 µl; Luciferin NT- 1 µl; Glutathione S-transferase- 1 µl) was added to the wells that contained the sample cells and the standards. Cells were then incubated at room temperature for 30 minutes in the dark (Figure 3.4). Following that, 50 µl of luciferin detection reagent was pipetted into each well.

Thereafter, the cells were incubated for 15 minutes. Luminescence was then measured. Luminescence was read on the Glomax® Discover Microplate Reader. GSH concentrations were determined by extrapolation on a standard curve and expressed as relative light units (RLU).

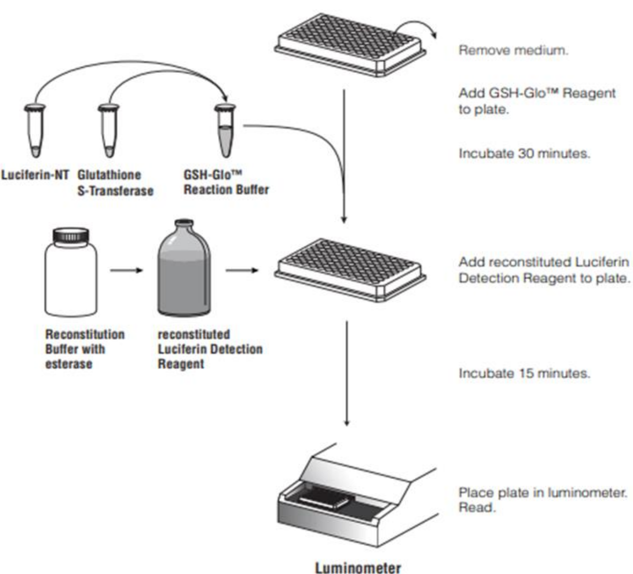

Figure 3.4: Treated MCF-7 breast cancer cells were exposed to GSH; thereafter, luminescence was measured (Bulletin 2015).

5.2 Quantification of reactive oxygen species

In the presence of moderately low levels of ROS, cancer cells activate signalling pathways that are important for cell survival and cell proliferation (Nakamura and Takada 2021). The intracellular redox state is determined by ROS levels, which were monitored by assessing the fluorescence levels using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescent probe. Dichloro-dihydro-fluorescein diacetate dye was utilised to measure the cell's redox state (Figure 3.5) (Eruslanov and Kusmartsev 2010). Monitoring the redox state of the cells can be conducted by detecting fluorescence (Eruslanov and Kusmartsev, 2010). Cells were centrifuged (400 x g for 10 min). The supernatant was removed, and cells were resuspended in 1 ml dye. Following that, cells were incubated for 45 minutes at 37 ºC. Thereafter, 100 µl PBS was added to the cells and centrifuged for 10 minutes. The cells were then washed three times with 1 ml PBS. Following the wash steps, the cells were then resuspended in 100 µl PBS. Subsequently, the flow cytometer Accuri™ C6 flow cytometer (BD Biosciences, USA) was used to measure the fluorescence (20,000 events) at 530 nm and the excitation at 485 nm.

Flow cytometry is a method used to measure fluorescence. It was utilised for detecting, identifying, and counting cells. Live cells were gated using the CFlow Plus Software (BD Biosciences, USA), and results were expressed as percentages.

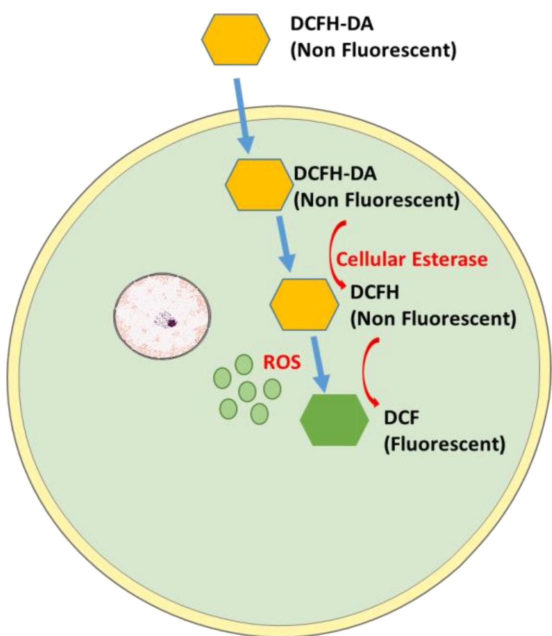

Figure 3.5: The DCFH-DA was taken up by the MCF-7 cells, where cellular esterase was cleaved off the acetyl groups, which resulted in DCFH. The DCFH was then oxidised by ROS and converted the molecule to DCF, which emitted green fluorescence (Kim and Xue 2020). Adapted from Abcam (2021). Figure created by Miss M Moremane using Microsoft Powerpoint 2013.

6. Assessment of Apoptosis

6.1. Caspase activity via Luminometry

Increased ROS, DNA fragmentation, and mitochondrial depolarisation activate apoptosis (Pfeffer and Singh 2018b). The activation of caspase-3 and 7 (effector caspases) and caspase-8 and 9 (initiator caspases) are needed for apoptosis (Brentnall et al. 2013). Apoptosis is induced when the initiator caspases are activated due to autocleavage. This will further activate the effector caspases that proteolysis substrates (Porter and Jänicke 1999; Slee, Adrain, and Martin 2001; Jan and Chaudhry 2019). Each assay was conducted as follows: Following treatment, 50 µl MCF-7 cells in PBS were pipetted into a white polystyrene 96-well microtitre plate in three replicates (Figure 3.6). Following that, 50 µl of the Caspase-

Glo®-3/7, Caspase-Glo® 8, and Caspase-Glo® 9 reagents, which were made according to the manufacturer's guidelines, were supplemented into each well. Cells were then incubated at room temperature for 30 minutes in the dark (Figure 3.6). Following incubation, the luminescence was determined on Glomax® Discover Microplate Reader. Fold change, and RLU was used to express the data.

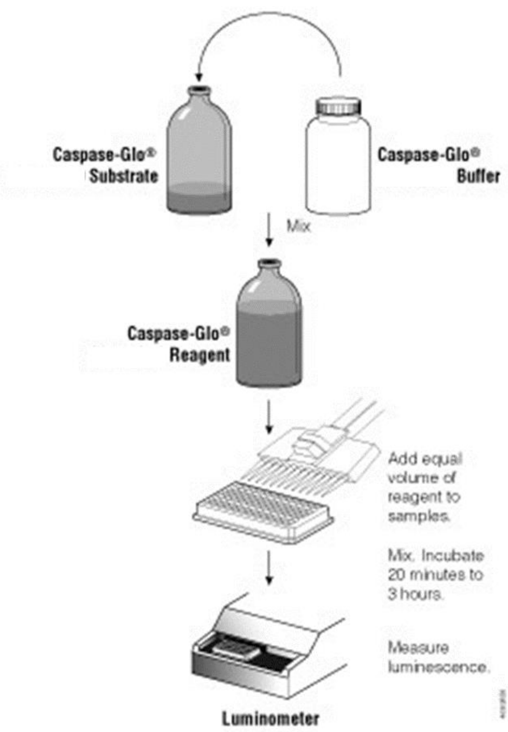

Figure 3.6: Schematic representation of caspase-3/7, -8 and -9 assay (Promega, 2022).

7. ATP levels via Luminometry

Metabolic activity was measured utilising the Cell-Titer Glo Assay (Promega) to quantify Adenosine triphosphate (ATP). ATP levels indicate the number of viable cells (Vialight 2011). The role of ATP, in this instance, is to allow apoptosomes, which are enzymes that activate caspases to induce cell death (Marek 2013). This method uses luciferase, an enzyme catalysing light formation (Figure 3.7) (Vialight 2011). Fifty microliters of MCF-7 cells (20,000) treated and resuspended in PBS were pipetted into a white polystyrene 96-well microtitre plate in three replicates. CellTiter-Glo® Reagent (50 µl) was added to the 96-well plate that already contained cells with 50 µl PBS. The plate was then incubated for 30

minutes at room temperature in the dark. Subsequently, luminescence was measured. A Glomax® Discover Microplate Reader was used to quantify the luminescence. The data expression was shown as RLU and fold change.

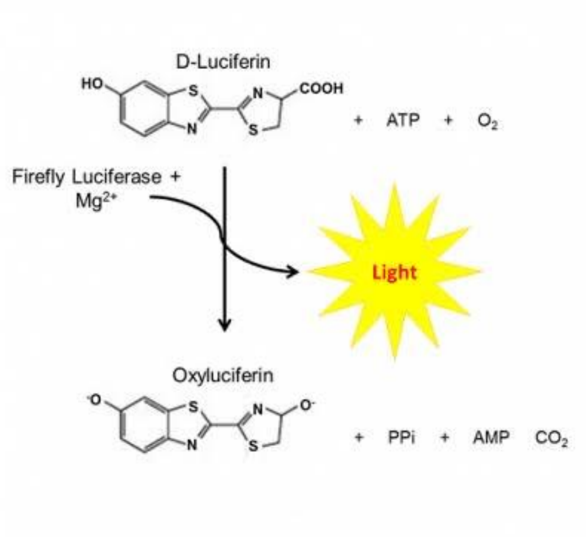

Figure 3.7: MCF-7 cells were exposed to treatment, and metabolic activity was assessed. This assay uses luciferase, an enzyme that catalyses light formation (Berthold 2022).

8. Western Blotting

8.1 Protein Isolation

Western blotting is a procedure used to detect and quantify protein expression. The identification of proteins such as Bax (inducer of apoptosis), Bcl-2 (inhibitor of apoptosis), p53 and Nrf2 is conducted to compare the expression of cell death. Additionally, a first responder in detecting DNA damage, PARP-1, was analysed as well. Briefly, proteins were separated depending on their molecular weight (Pillai-Kastoori, Schutz-Geschwender, and Harford 2020). Following treatment, proteins were isolated by initially washing the cells with 1 ml PBS. After that, 300 µl of the CelLyticTM M extraction reagent solution (catalogue number C2978) was added to the cells and incubated for 15 minutes on a shaker. Following incubation, the cells were removed from the flasks (scraping the cells from the flasks increased the protein yield). The lysed cells were therefore centrifuged for 15 minutes at 5000 x g. Subsequently, the supernatant containing the proteins was added to a chilled microtube.

8.2 Protein Quantification

The microtubes that previously contained protein were thawed on ice. Following that, standards and the working reaction were prepared. In order to prepare the standards and the working reaction, PBS was used as a diluent. One Bovine Serum Albumin (BSA) standard ampule was used to prepare the standards into several eppendorfs with PBS as a diluent utilising the manufacturer's guidelines (Table 3.1).

Table 3.1: The preparation of Bovine Serum Albumin (BSA) standards that are diluted per the manufacturer's guidelines.

Vial	Dilution Scheme for Standard Microplate Procedure (Working Range = 20 – 2,000 µg/mL)	Diluent volume (PBS) (µL) and Source volume (BSA) (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25

I	400	0	1 = Blank

The proteins were then quantified and standardised to 1000 ng/µl concentration utilising Qubit 4, ThermoFisher Scientific, SA. Each standard and unknown sample of 25 µl was pipetted into wells in triplicates. A working reaction of 200 µl was added to each well. Working reaction comprised a mixture of Bicinchoninic Acid (BCA) reagent A (50 parts) and BCA reagent B (1 part). The plate was mixed thoroughly, utilising a plate shaker for 30 seconds. After that, a clear 96-well plate was covered in foil and incubated for 30 minutes at 37℃. Absorbance was then measured at 562 nm using the GloMax plate reader machine after being cooled at room temperature.

8.3 Preparation of protein for SDS-PAGE

A Laemmli buffer (2X) and 2-Mercaptoethanol were mixed and added to the standardised protein in a ratio of 1:1. The prepared samples were exposed to heat (100 ℃) for five minutes using a heat block.

8.4 SDS-PAGE

The 10% SDS-Page gels were assembled into the electrode assembly with the short plates facing inward. The gel cassette was placed into the SDS-Page tank, and the inner chamber was then filled with a cold SDS-Page running buffer (1X). Subsequently, the green combs were removed from the gel and the molecular weight marker, and the protein samples were vortexed. The protein loading tips and a loading guide were utilised to add protein samples of 28 µl to the gel wells. A molecular weight marker of 10 µl was also supplemented to the wells in the gel. After loading all samples into the gel, the inner chamber was topped up with a cold SDS-Page running buffer (1X). Following that, the outer chamber was also filled with an SDS-Page running buffer (1X). The SDS-Page tank was placed in a dish that had ice surrounding it, and a lid was therefore placed on the SDS-Page tank. After that, the SDS-Page tank was connected to the power pack. The SDS-Page gel electrophoresis was then run at 150V for an hour.

8.5 Transferal of SDS-PAGE

Following the SDS-Page gel electrophoresis, a container was filled with a transfer buffer. The gels, the plastic plate, and the stacking gel were removed from the SDS-Page tank. Then, the migrated protein was added with a resolving gel to a container with a transfer buffer and was

allowed to equilibrate for a few minutes. The gel was transferred onto a nitrocellulose membrane utilising the TransBlot Turbo transfer system (BioRad) (25V, 30 minutes) (Figure 3.8). The membrane was blocked using 5% BSA in Tris-buffered saline that contained 0,05% tween-20 (TTBS) at room temperature on a shaker for an hour and then incubated with primary monoclonal antibodies (5 µl): p53 (53 kDa) (7F5) (Rabbit mAb cat. no. 2527T); Bcl-2 (26 kDa) (124) (Mouse mAb cat. no. 15071); PARP-1 (116.89 kDa) (46D11) (Rabbit mAb cat. no. 9532T); Bax (20 kDa) (Rabbit mAb cat. no. 2772T); and Nrf2 (97-100 kDa) (D1Z9C) XP (Rabbit mAb cat. no. 12721T) overnight at 4℃ on an Oncoshaker. Following the overnight incubation, the membranes were placed on a shaker for an hour at room temperature to equilibrate. The membrane was then washed (4x) with TTBS for 15 minutes each. After the washing of the membranes, horseradish peroxidase-conjugated secondary antibody (1:5000); anti-rabbit HRP-linked IgG (cat. no. 7074S); anti-mouse HRP-linked IgG (cat. no. 7076S) were prepared in 5 ml of 5% BSA in TTBS and incubated for one hour on a shaker at room temperature. The membranes were rinsed with TTBS four times for 15 minutes each and, therefore, rinsed with dH_2O after the fourth TTBS wash. The dH_2O was then discarded, and the membrane was placed on transparency. Detection of protein bands was conducted using 300 µl Clarity Western ECL viewing substrate detection kit Bio-Rad (USA). The reagents were mixed and added to cover the membrane. Following that, the membrane was then placed in a ChemiDoc and was viewed using the chemiluminescence protocol.

The membranes were re-probed by adding TTBS wash buffer to them for rinsing. Then, 5 ml stripping buffer was added to the membrane to remove the bound antibody. Following that, the membrane was incubated at room temperature conditions on a shaker for 30 minutes. After incubation, the stripping buffer was discarded, and TTBS wash buffer (1X) was added to rinse the membrane for 15 minutes. The TTBS wash buffer was removed, and 5 ml of 5% BSA in TTBS was added to cover the nitrocellulose membrane. The membrane was blocked by being placed on a shaker for 30 minutes. Fresh 5 ml of 5% BSA in TTBS was added to cover the nitrocellulose membrane.

For normalisation, the membrane was re-probed with β-actin (42 kDa) (5 ml of 5% BSA in TTBS) (D6A8) (Rabbit mAb cat no. 8457). The membrane was incubated on a shaker at room temperature for 30 minutes. Then, TTBS wash buffer was used to rinse the membrane twice for 15 minutes each. The wash buffer was then discarded, and the membrane was

washed twice for 15 minutes, each with deionised water. The signal was detected using the ChemiDoc imaging system (BioRad) (Figure 3.8). Protein expression of Bcl-2, PARP-1, Nrf2, p53, and Bax was examined utilising the BioRad image lab software. The data was expressed as relative band density and fold change (Tiloke, Phulukdaree, and Chuturgoon 2013). The reagents of western blot were procured from Bio-Rad (USA).

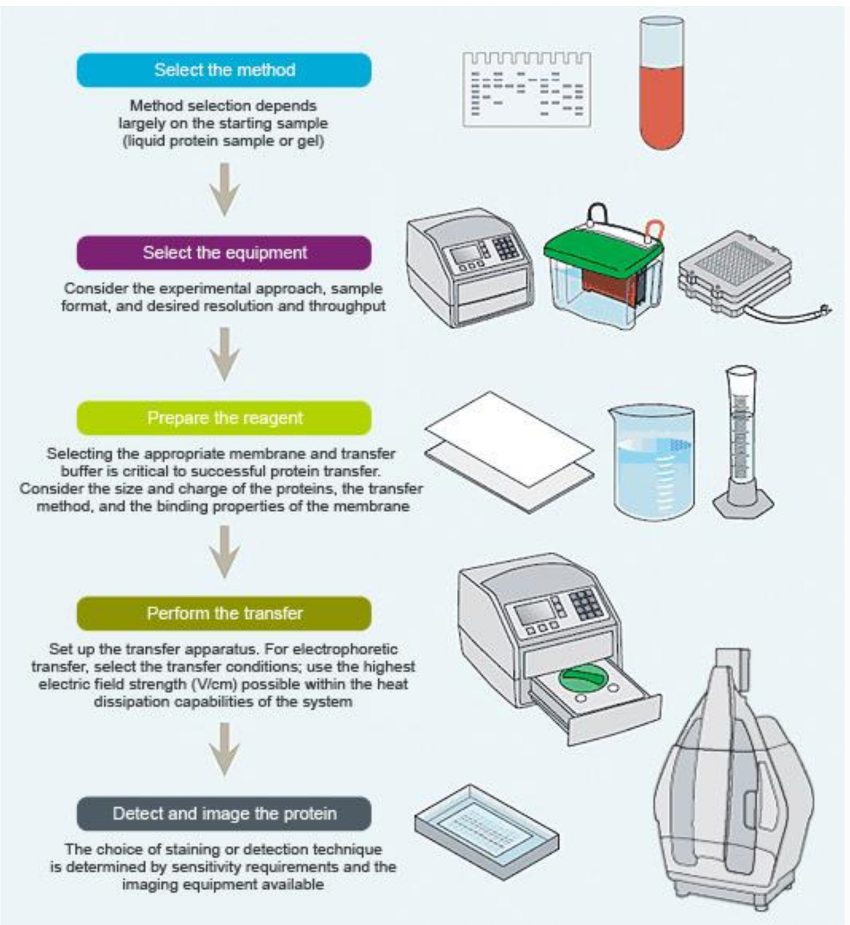

Figure 3.8: This procedure detected and quantified the protein expression of MCF-7 treated cells (Bio-Rad 2022).

8.6 Protein Band Intensity quantification (Densitometry)

The protein bands in the obtained images were quantified using the Image Lab software (Bio-Rad Laboratories, Inc., Version 6.1.0 build 7). They were refined in terms of display functionality. The intensity levels of the proteins of interest, were normalized to the intensity of the β-actin loading control. These normalised protein intensity measurements were then exported to Microsoft Excel for further processing before being statistically analysed using the necessary tests.

9. Quantitative Polymerase chain reaction (qPCR) – mRNA expression

9.1 The RNA extraction

The quantitative polymerase chain reaction is a method used to measure mRNA levels. It is utilised for detecting, characterising, and quantifying nucleic acids (Figure 3.9) (Kadri 2019). As explained in the manufacturer's guideline, the expression of the mRNA of Nrf2 and p53 was determined by initially isolating RNA from the control and the treated cells. Cells were washed with PBS three times. Then, Tri reagent and PBS with a volume of 500 µl each were added. After that, cells were incubated for five minutes at room temperature in the dark. The RNA was then removed from each flask by scrapping and adding 1 ml into eppendorfs.

9.2 The RNA purification

The purification of proteins was done by adding 1 ml of ethanol to the supernatant that contains the proteins. The mixture was then transferred into the Zymo-spin ™ IICR column in a collection tube and centrifuged at 12 000 x g for 30 seconds. Following that, the column was transferred into a clean collection tube. The flow-through was then discarded.

Subsequently, an RNA wash buffer of 400 µl was added to the column for the DNAse I treatment and centrifuged for 30 seconds at 12 000 x g. A mixture of 5 µl DNAse I and 75 µl DNA digestion buffer (in an RNase-free tube) was added directly into the column matrix and was incubated for 15 minutes at RT.

Direct-zol ™ RNA prewash (400 µl) was added to the column and centrifuged at 12 000 x g for 30 seconds. The column was then transferred into a new collection tube. The step was repeated two times. Then, RNA wash buffer (700 µl) was added to the column and centrifuged for one minute at 12 000 x g. The content in the column was then transferred into a clean RNase-free tube. The RNA was then eluted by adding 50 µl of DNase/RNase-free water directly to the column matrix and centrifuged for 30 seconds at 12 000 x g.

9.3 Quantification of RNA

At room temperature, the RNA samples were thawed for the QubitTM RNA HS assay. Solutions of the Qubit™ RNA IQ assay were also thawed at room temperature. The working solutions of the Qubit™ were prepared by diluting the Qubit™ RNA IQ reagent 1:200 in Qubit™ RNA buffer utilising the conical tube of 50 ml. A working solution of Qubit™ RNA (190 µl) was added to each tube used for the standards. Subsequently, 10 µl of each Qubit™ standard was added to the tube and vortexed for two to three seconds. The tubes were then

incubated for two minutes, and a Qubit™ 4 Fluorometer was used to read the samples and standards.

9.4 The integrity and quality of RNA

The Qubit™ working solution was prepared by diluting the Qubit™ RNA IQ reagent in a Qubit™ RNA IQ buffer. A working solution of 190 µl Qubit™ was added to each tube utilised for standards. Following that, 10 µl Qubit™ standards were added to each appropriate. The mixture was vortexed for two to three seconds, avoiding bubble creation. The tubes were incubated for two minutes at room temperature. The purity of RNA was determined at the absorbance ratio of A260/A280.

9.5 High-capacity RNA-to-cDNA™

The high-capacity RNA-to-cDNA™ kit (Thermofisher Scientific, SA) (cat. no. 4387406) was used to reverse transcribe RNA into copy/complementary DNA (cDNA) as described by the manufacturer's instructions. In brief, a 20 µl reaction was prepared by supplementing 1 µl of iScript reverse transcriptase, 4 µl of 5X iScript reaction mix, RNA template (2000 ng), and nuclease-free water. The end-product was subjected to a temperature of 25ºC for five minutes, 42ºC for 30 minutes, and 85ºC for five minutes and a final hold at 4ºC using a ProFlex Themocycler to obtain cDNA.

10. Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR (qPCR) utilising the iQ Superscript reagent (Bio-Rad, SA) was conducted to determine the expression of mRNA (Figure 3.9). A reaction mix of 9 µl was added to each well in the qPCR plate, followed by 1 µl of cDNA into each well. A blank sample was also added to the plate. The reaction of 25 µl that consisted of the IQ™ SYBR green supermix of 12,5 µl, 0.1 x TE buffer (Lot: 07.10.2021) of 8,5 µl, cDNA of 2 µl, as well as 1µl sense and anti-sense primer (Table 3.2), was utilised (Juhasz et al. 2003).

The mRNA expression was evaluated and normalised to *GAPDH*, a housekeeping gene that compares the gene expression data (Barber et al. 2005). An initial denaturation of 95ºC for 10 minutes was used. Subsequently, 40 cycles followed at 95ºC for 15 seconds (denaturation), annealing (40 seconds) (Table 3), and extensions (72ºC for 30 seconds) (QuantStudio™ 5 Real-Time PCR System, Applied Biosystems) (Figure 3.9). The expression of mRNA was

measured utilising the Livak method and was expressed in fold changes relative to the control (Livak and Schmittgen 2001).

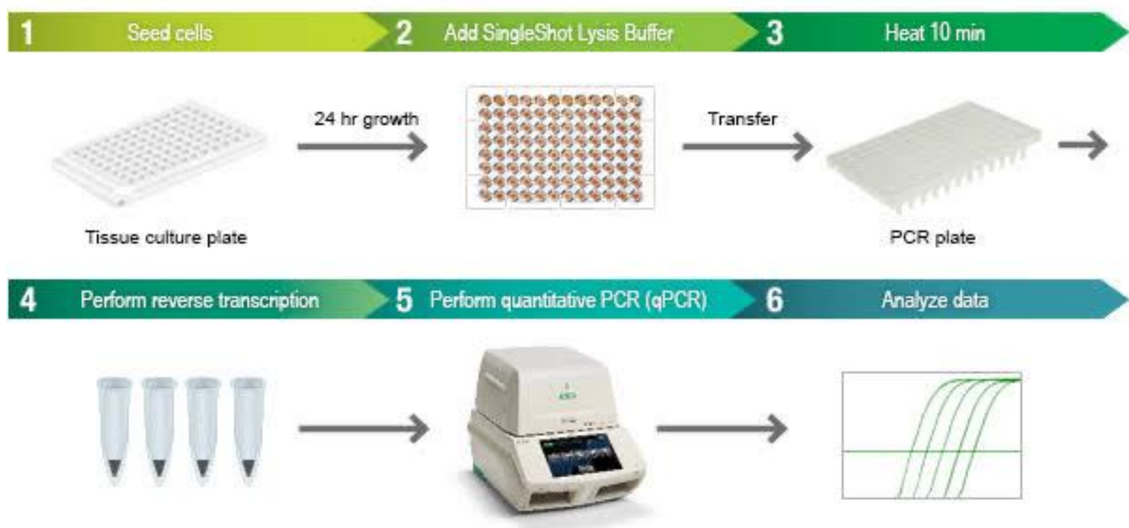

Figure 3.9: The qPCR procedure measures mRNA levels. It is used for detecting, characterising, and quantifying nucleic acids (Weebly 2023).

Table 3.2: Primer sequences

Sequence of primers			**Annealing temperature (ºC)**	**References**
	Sense	**Anti-sense**		
p53	5'-CCACCATCCACTACAACTACAT-3'	5'-AGGAAGCGGTCCAGGTAGTT-3'	56	(Long and Wu 2008)
Nrf2	5′ -AGTGGATCTGCCAACTACTC - 3'	5′ -CATCTACAAACGGGAATGTCTG- 3'	58	(Naidoo et al. 2017; Lo and Matthews 2013)

GAPDH	5’-TCCACCACCCTGGTTGCTGTA-3’	5’-ACCACAGTCCATCCCACAC-3’	56/58	(Tiloke, Phulukdaree, and Chuturgoon 2013; Nazari et al. 2015)

11. Statistical Analysis

The data collected was quantitative data. Graph Pad Prism v.7.0 software was utilised to analyse the data (Graph Pad Software Inc., San Diego, California USA). The mean and standard deviation (SD) were used to express the results. The experiments were repeated 3 times with 3 replicates. For the IC_{50} determination from the MTT assay, a dose-response-inhibition equation was performed by non-linear regression analysis, using the [Inhibitor] vs. normalized response - variable slope template. Statistical analysis was conducted using One-way ANOVA analysis and a 95% confidence interval. The data was regarded as statistically significant when the value of $p < 0.05$.

CHAPTER 4: RESULTS

4.1 Introduction

Chapter 4 describes the results of the quantitative data obtained using the methodology outlined in Chapter 3. The MCF-7 breast cancer cells were exposed to MO and Dox. The treatment groups for all experiments included Dox as a positive control, MO as an individual treatment to assess its antiproliferative potential and the combination of MO and Dox. Biochemical analysis was conducted: methyl thiazoltetrazolium (MTT) assay to assess cell viability, glutathione (GSH) assay to quantify oxidative stress, dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay to quantify reactive oxygen species, and caspase activity assay was conducted to assess apoptosis. In addition, adenosine triphosphate (ATP) assay as a measure of metabolic activity, western blotting procedure (protein expression), and quantitative polymerase reaction (qPCR) for mRNA expression were also performed

4.2 Cytotoxicity Assay

4.2.1 Effect of *Moringa oleifera* on cell viability of MCF-7 cells

The half maximum inhibitory concentration (IC_{50}) of MO was determined. The cells were exposed to dose concentrations (0, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg/ml) for 24, 48 and 72 hours, and cell viability was measured using an MTT assay. The statistical software programme GraphPad Prism version 9.4.0 using the non-linear regression model was used to determine the IC_{50} value. A decrease in MCF-7 cell viability was observed in cells treated for 24, 48 and 72 hours of treatment (Appendix A). Based on the results, 2600 µg/ml was the most effective concentration in reducing the live cells to below 50% compared to the control and used in all subsequent experiments for 72 hours (Figure 4.1).

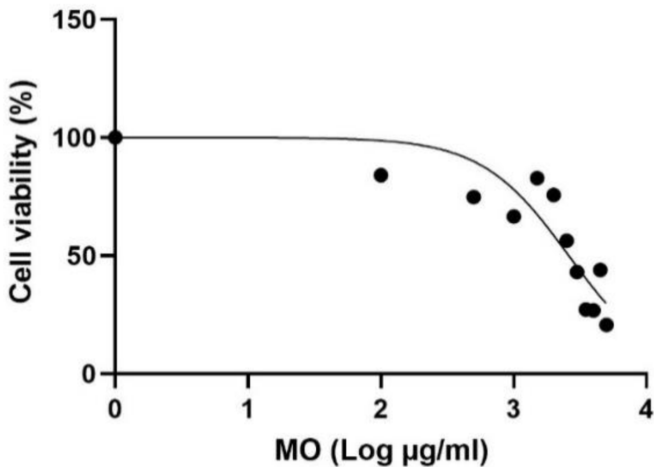

Figure 4.1: Dose-response curve following the administration of MO in MCF-7 cells for 72 hours. Please refer to the 24- and 48-hour treatment results in Appendix A.

4.2.2 Effect of Doxorubicin treatment on cell viability of MCF-7 cells

The cellular growth inhibitory effect of Dox and the effective dose for 50% cell survival (IC_{50}) was determined. The MCF-7 cells were treated with log_{10} increasing concentrations (0.01, 0.1, 1 and 10 µM) for 24, 48 and 72 hours. Cell viability was then assessed following drug treatment. There was decreased viability observed in the cells treated for all three periods. The results demonstrated a dose and time-dependent decrease in cell viability. The viable cells were reduced to below 50% at the 72-hour period (Figure 4.2). Therefore, the most effective time frame was 72 hours at an IC_{50} concentration of 0.978 µM, which was used for all subsequent experiments.

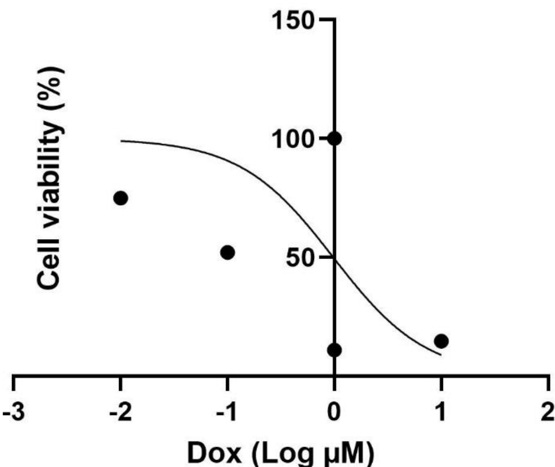

Figure 4.2: Dose-response curve following the treatment of Dox on MCF-7 cells for 72 hours. Please refer to the 24- and 48-hour treatment results in Appendix B.

4.3 Oxidative stress determination assay

Oxidative stress was assessed using the glutathione assay. *Moringa oleifera* (MO) significantly decreased the total glutathione levels (GSH) in the MCF-7 breast cancer cells when compared to the control (5 ± 0.05 RLU vs 6.3 ± 0.45 RLU, $p < 0.0365$). Compared to the control, a significant increase in GSH levels was observed in Dox treated cells (11 ± 0.37 RLU vs 6.3 ± 0.45 RLU, $p < 0.0002$). However, the combination of MO + Dox presented with marginally decreased GSH levels following a 72-hour treatment (6.1 ± 1.08 RLU vs 6.3 ± 0.45 RLU, $p < 0.9999$) (Figure 4.3).

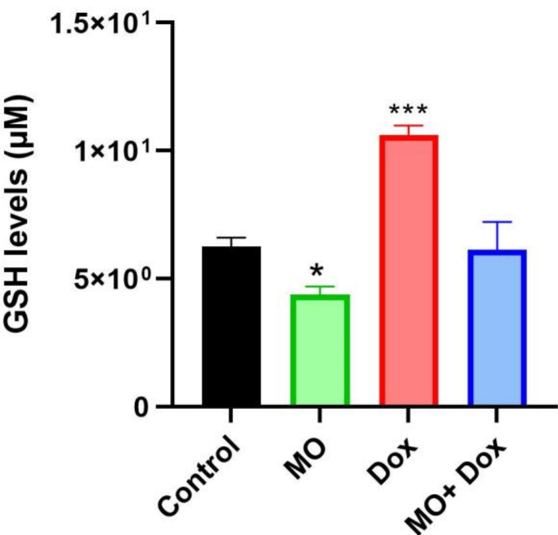

Figure 4.3: The effect of MO and Dox on the endogenous levels of glutathione. MCF-7 cells were treated with Dox (0.978 µM) and MO (2600 µg/ml) for a period of 72 hours, and for the same duration, the cells were subjected to the combination of MO + Dox. Significance is indicated as * ($p < 0.05$); *** ($p < 0.001$) (n=3).

4.4 Quantification of Reactive Oxygen Species

ROS production was assessed using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescent dye. Cellular ROS production was elevated following MO treatment relevant to the untreated control (35 ± 8.46 RLU vs 30 ± 8.47 RLU, $p < 0.9999$). A similar trend of elevated ROS levels was noticed following Dox treatment (38 ± 13.88 RLU vs 30 ± 8.47 RLU, $p < 0.9999$) as well as the combination of Dox and MO when compared to the control (40 ± 14.61 RLU vs 30 ± 8.47 RLU, $p < 0.9999$).

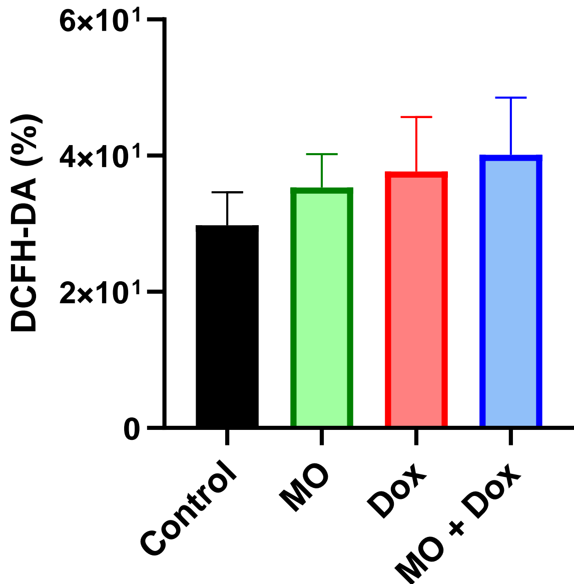

Figure 4.4: The effect of *Moringa oleifera* (MO) and Dox on the ROS production in MCF-7 cells. A dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescent dye was used for the assessment of ROS generation in cells exposed to Dox (0.978 µM) and MO (2600 µg/ml) as well as cells that were co-treated with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours (n=3).

4.5 Assessment of Apoptosis

4.5.1 Caspase activity via Luminometry

4.5.1.1 Caspase-9 activity

Caspases are known as the main mediators of apoptosis (Aral, Aral, and Kapila 2019). Caspase-9 is described as the initiator of the intrinsic pathway of cell death. Therefore, MCF-7 cells were exposed to the Caspase-9 reagent for cell elimination. This was through the execution of early apoptotic death in the stage of development. Caspase-9 was elevated significantly in cells treated with MO (6028000 ± 66505.64 RLU vs 2596333 ± 132001.26 RLU, $p < 0.0001$). Moreover, the cells treated with Dox as a mono-treatment also showed a significant increase in apoptosis when compared to the control (10123667 ± 10937891.59 RLU vs 2596333 ± 132001.26 RLU, $p < 0.0213$). Similarly, it was observed that the co-treatment of Dox and MO led to a significant increase in cell death when compared to the

control (7895667 ± 238363.87 RLU vs 2596333 ± 132001.26 RLU, $p < 0.0001$) (Figure 4.5.1.1).

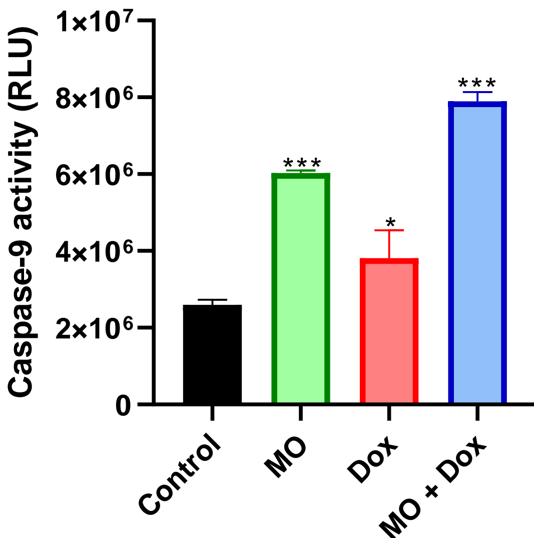

Figure 4.5.1.1: The effect of MO and Dox on apoptosis in MCF-7 cells. Caspase-9 reagents were used to measure cell death in cells exposed to Dox (0.978 µM) and MO (2600 µg/ml) as well as cells treated with both MO (2600 µg/ml) and Dox (0.978 µM) for 72 hours. Significance is indicated as * ($p < 0.05$); *** ($p < 0.001$) (n=3).

4.5.1.2 Caspase-8 activity

Caspase-8 enzyme activity is responsible for initiating the death receptor pathway of apoptosis in the MCF-7 cells. Caspase-8 is also known to mediate the extrinsic apoptotic pathway (Aral, Aral, and Kapila 2019). It plays a significant role in promoting the death of the cells. The cells exposed to MO as a mono-treatment showed a significant increase in the activity of the initiator caspases compared to the control (3360333 ± 75745.19 RLU vs 1425333 ± 30171.73 RLU, $p < 0.0001$). Similarly, the mono-treatment of Dox led to a significant increase in cell death compared to the control (1776000 ± 39962.48 RLU vs 1425333 ± 30171.73 RLU, $p < 0.003$). Moreover, the co-treatment of Dox and MO showed a similar trend of significantly increasing apoptosis in the MCF-7 cells when compared to the control (41973.33 ± 60871.45 RLU to 1425333 ± 30171.73 RLU, $p < 0.0001$) (Figure 4.5.1.2).

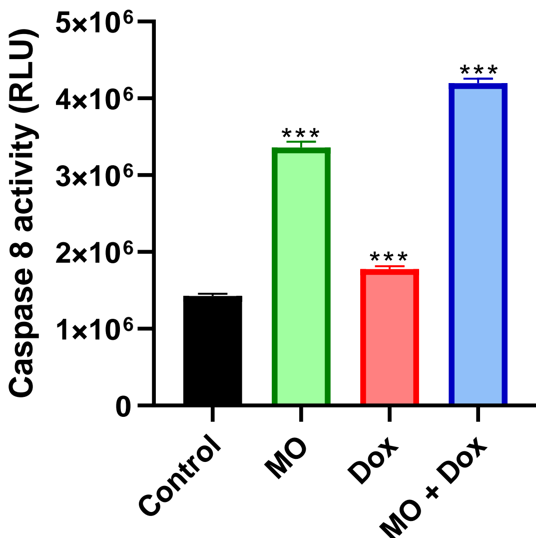

Figure 4.5.1.2: The effect of MO and Dox on the induction of apoptosis in MCF-7 cells. Caspase-8 reagent was used to assess cell death in cells treated with Dox (0.978 µM) and MO (2600 µg/ml) as well as cells that were co-treated with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours. Untreated cells served as the control. Significance is indicated as *** ($p < 0.001$) (n=3).

4.5.1.3 Caspase-3/7 activity

Caspase-3/7 reagent was utilised to measure apoptosis. Caspase-3 plays a significant role in apoptosis by destructing cellular structures such as DNA fragmentation, inducing ROS production, thus efficiently killing the cells, whilst caspase-7 leads to loss in cell viability. The cells treated with MO demonstrated a significant increase in the activity of effector caspases compared to the control (313000 ± 14471 RLU vs 79026, 67 ± 7199, 2 RLU, $p < 0.0001$). Similarly, Dox exposure to the cells showed a significant increase in activity compared to the control (760900 ± 18147.18 RLU vs 79026.67 ± 7199.2 RLU, $p < 0.0001$). Moreover, cells that were exposed to the combination of MO plus Dox also increased the level of apoptosis significantly in the MCF-7 cells when compared to the control (1154666.67 ± 3055. 05 RLU to 79026.67 ± 7199.2 RLU, $p < 0.0001$) (Figure 4.5.1.3).

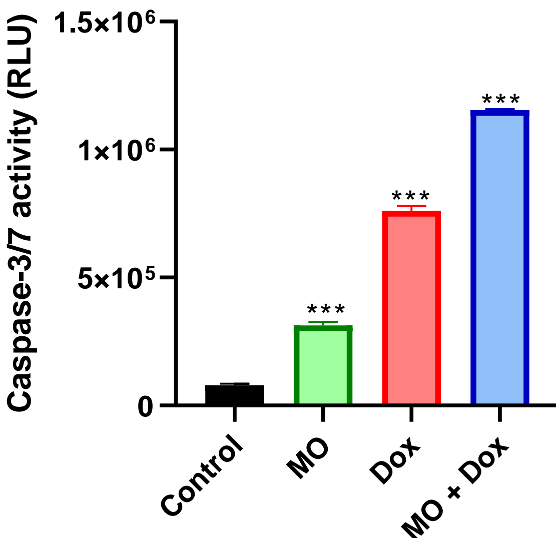

Figure 4.5.1.3: The effect of MO and Dox on the execution of apoptosis in MCF-7 cells. Caspase-3/7 reagent was used to appraise cell death in cells exposed to Dox (0.978 µM) and MO (2600 µg/ml) as well as cells that were co-treated with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours. Significance is indicated as *** ($p < 0.001$) (n=3).

4.6 ATP levels via Luminometry

The effect of MO on metabolic activity was assessed utilising the ATP assay on MCF-7 cells. ATP is known as the energy currency for cells. Moreover, cells have been shown to die with elevated ATP during apoptosis (Zamaraeva et al. 2005). A significant increase in metabolic activity in cells treated with MO was displayed compared to the control (8721000 ± 193651.75 RLU vs 5649667 ± 233840.40 RLU, $p < 0.0001$). Furthermore, the combination of MO plus Dox also showed a significant increase in metabolic activity of the MCF-7 cells when compared to the untreated control (9295000 ± 116051.71 RLU to 5649667 ± 233840.40 RLU, $p < 0.0001$). However, a modest decrease in metabolic activity was observed in cells exposed to Dox (5276667 ± 354505.76 RLU vs 5649667 ± 233840.40 RLU, $p < 0.5641$) when compared to the control (Figure 4.6).

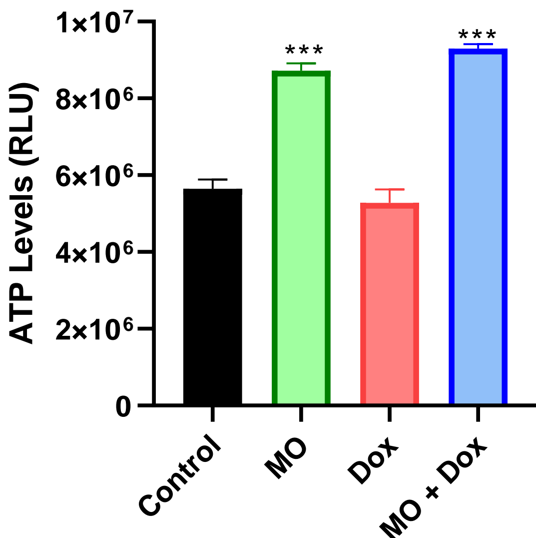

Figure 4.6: The effect of MO and Dox on MCF-7 breast cancer cells resulted in increased metabolic activity (ATP levels). The MCF-7 breast cancer cells were introduced to Dox (0.978 µM) and MO (2600 µg/ml) and were also co-exposed with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours. The untreated cells served as a control. Significance is indicated as *** ($p < 0.001$) (n=3).

4.7 Western Blot Analysis

4.7.1 Preliminary expression of Nrf2

The Nrf2 protein is responsible to defend the cell against oxidative damage. The Nrf2 protein prevents carcinogenesis by suppressing ROS and leading to oxidative damage repair through its target gene expression (Rojo de la Vega, Chapman, and Zhang 2018). There was a decrease in the expression of Nrf2 by 0.614 fold change in MO-treated MCF-7 cells compared to the untreated cells. Additionally, when cells were exposed to Dox, the Nrf2 expression decreased compared to the control (0.258 fold change). Moreover, the combination treatment, Dox and MO, decreased the expression of Nrf2 in the MCF-7 cells (0.788 fold change) (Figure 4.7.1).

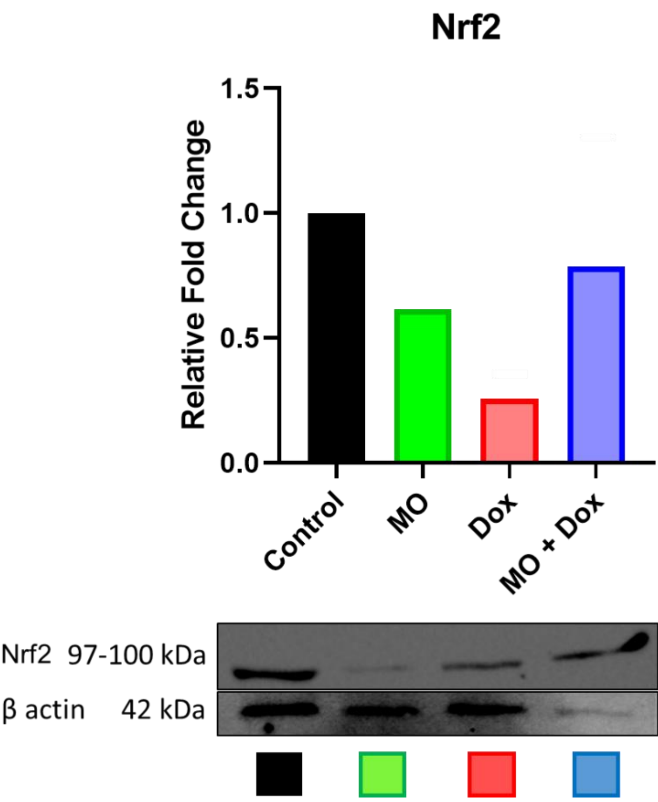

Figure 4.7.1: The effect of MO and Dox on Nrf2 protein expression in MCF-7 cells. Nrf2 was used to assess oxidative damage in cells exposed to a control, Dox (0.978 µM) and MO (2600 µg/ml), as well as cells that were co-treated with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours (n = 1).

4.7.2 Preliminary expression of p53

The tumour protein p53 is known as the tumour suppressor protein. It is essential for preventing cancer development by regulating the repair of DNA as well as cellular division (Ozaki & Nakagawara, 2011). The expression of p53 in the MCF-7 cells was highly upregulated in the MO-treated cells by 4.345 fold change compared to the control. A similar observation was seen on Dox-exposed MCF-7 cells, where the p53 levels were slightly expressed compared to the control (1.065 fold change). Moreover, when cells were treated with the combination of MO + Dox, there was a slight upregulation of the p53 protein in the MCF-7 cells compared to the control (1.026 fold change) (Figure 4.7.2).

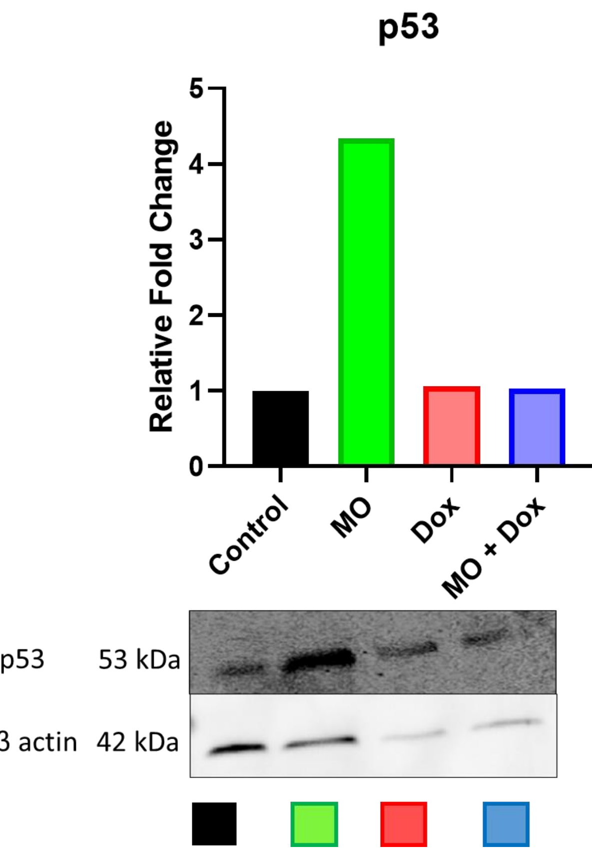

Figure 4.7.2: The effect of MO and Dox on p53 protein expression in MCF-7 cells. The researcher used p53 to assess cell death in cells exposed to a control, Dox (0.978 µM) and MO (2600 µg/ml), as well as cells that were co-administered with MO (2600 µg/ml) + Dox (0.978 µM) for a 72 hour period (n = 1).

4.7.3 Preliminary expression of Bcl-2

The Bcl-2 gene from the family of Bcl-2 is an anti-apoptotic (pro-survival) protein. It plays a role in inhibiting apoptosis, resulting in the survival of cancer cells even under stressful conditions. The Bcl-2 expression in the MO-treated cells was downregulated when compared to the control (0.205 fold change). A similar expression was observed when cells were exposed to Dox, which showed low levels of Bcl-2 compared to the control cells (0.047 fold change). Cells treated with the MO + Dox showed a decrease in the Bcl-2 expression compared to the control (0.099 fold change) (Figure 4.7.3).

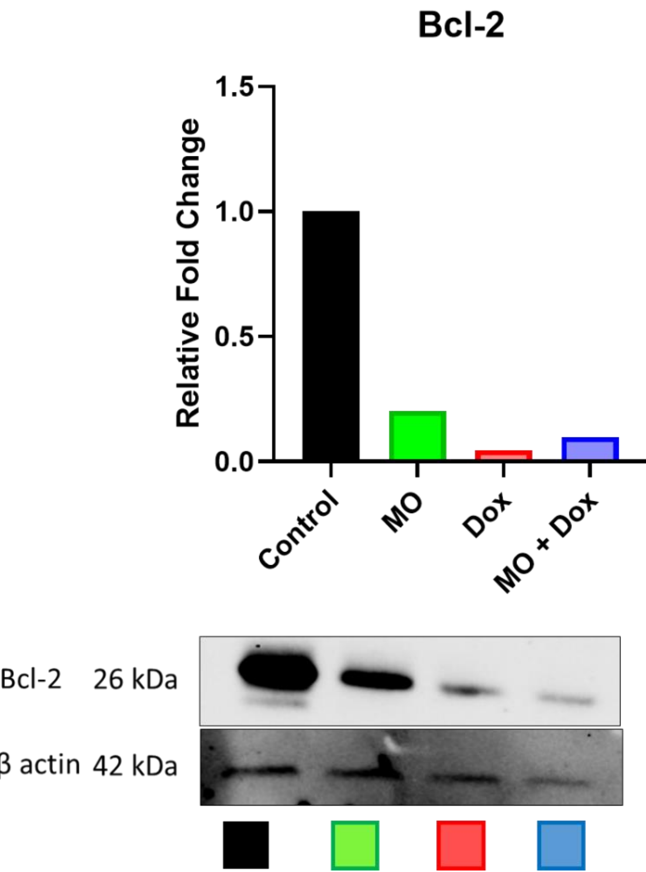

Figure 4.7.3: The effect of MO and Dox on Bcl-2 protein expression in MCF-7 cells. Bcl-2 was used to assess the inhibition of apoptosis in cells exposed to a control, Dox (0.978 µM) and MO (2600 µg/ml), as well as cells that were co-treated with both MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours (n = 1).

4.7.4 Preliminary expression of Bax

Bax is a pro-apoptotic protein that forms part of the Bcl-2 family of proteins. It is essential in promoting apoptosis by initiating mitochondrial membrane permeabilisation. The exposure of MO to the MCF-7 cells led to a 0.317 fold change decrease in Bax expression compared to the control. A similar observation was seen on the Dox-treated cells, with low levels of Bax in the MCF-7 cells relative to the control (0.114 fold change). Moreover, the combination treatment of MO + Dox also showed a decrease in Bax levels in the MCF-7 treated cells compared to the control (0.097 fold change) (Figure 4.7.4).

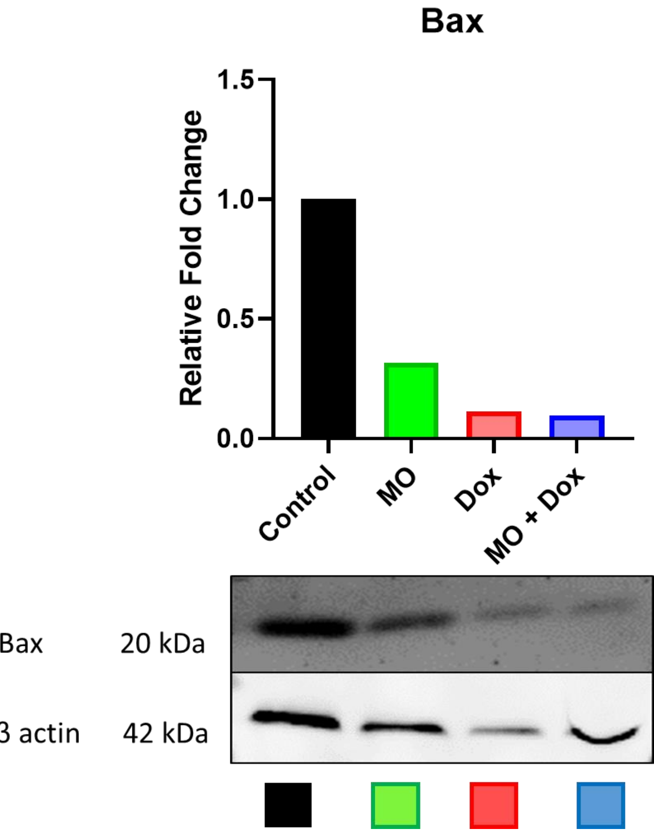

Figure 4.7.4: The effect of MO and Dox on Bax protein expression in MCF-7 cells. Bax was used to assess cell death in cells exposed to a control, Dox (0.978 µM) and MO (2600 µg/ml), as well as cells that were co-treated with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours (n = 1).

4.7.5 Preliminary expression of PARP-1

The nuclear enzyme PARP-1 is a protein that responds to DNA damage. It is essential for DNA repair in cells. The PARP-1 expression in MO-exposed cells was downregulated by 0.053 fold change compared to the untreated cells. Moreover, the same observation was seen in cells treated with Dox, which showed low expression of PARP-1 in the MCF-7 cells (0.031 fold change). When cells were co-exposed to the combination treatment, MO + Dox, there was downregulation of the PARP-1 protein compared to the control (0.472 fold change) (Figure 4.7.5).

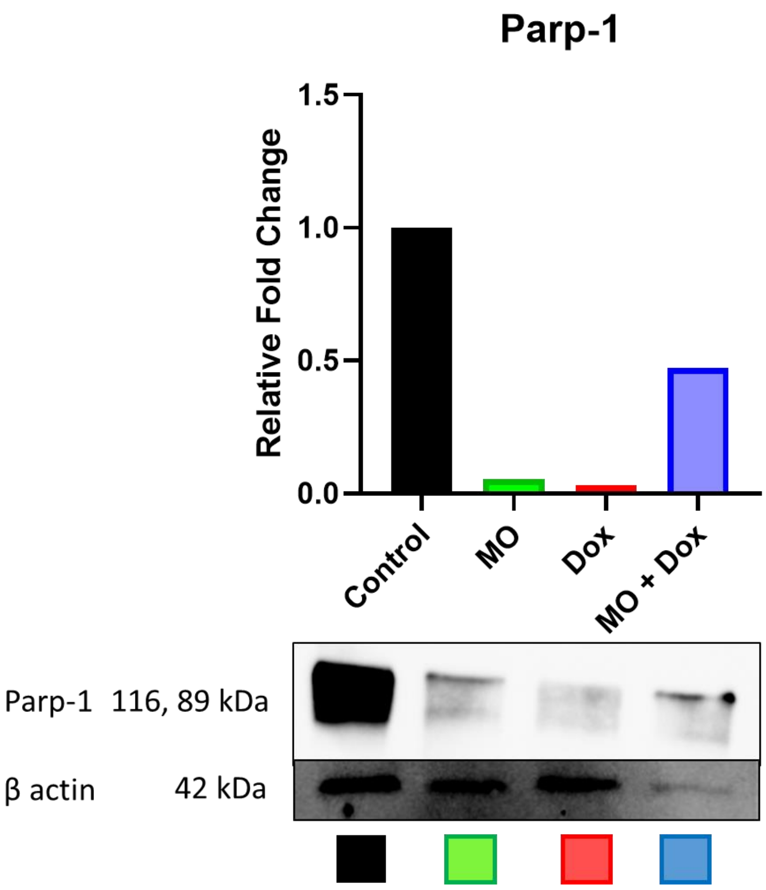

Figure 4.7.5: The effect of MO and Dox on the PARP-1 protein expression in MCF-7 cells. PARP-1 was used to assess DNA repair in cells exposed to a control, Dox (0.978 µM) and MO (2600 µg/ml), as well as cells that were co-treated with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours (n=1).

4.8 qPCR analysis

4.8.1 *p53* mRNA expression

Tumour protein p53 (also known as p53) is a protein encoded by *TP53* gene. This protein controls cell division by acting as a tumour suppressor, which prevents cells from growing and dividing too quickly or in an uncontrolled manner (Ozaki & Nakagawara, 2011). The mRNA expression of *p53* in the MCF-7 cells was downregulated in the MO-treated cells by 0.71 fold change compared to the control. A similar observation was seen in Dox-exposed

MCF-7 cells, where the *p53* levels were decreased compared to the control (0.87 fold change). Moreover, when cells were treated with the combination of MO + Dox, there was downregulation of the *p53* mRNA in the MCF-7 cells compared to the control (0.65 fold change) (Figure 4.8.1).

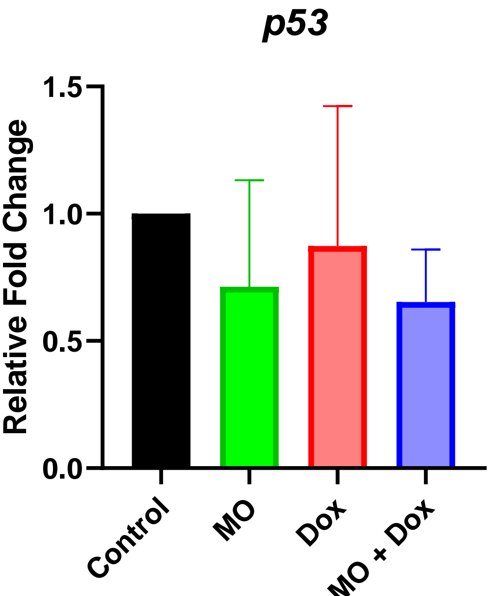

Figure 4.8.1: The effect of MO and Dox on the p53 mRNA expression in MCF-7 cells. *p53* mRNA expression was used to assess cell death in cells exposed to a control, Dox (0.978 µM) and MO (2600 µg/ml), as well as cells that were co-administered with MO (2600 µg/ml) + Dox (0.978 µM) for a 72 hour period (n = 3).

4.8.2 *Nrf2* mRNA expression

Transcriptional regulator Nrf2 is a crucial component of antioxidant defence. By interacting with the antioxidant response elements, it promotes the transcription of cytoprotective genes that produce antioxidants, such as GSH (Kavian et al. 2021). There was a decrease in the expression of *Nrf2* by 0.44 fold change in MO-treated MCF-7 cells compared to the untreated cells. However, when cells were exposed to Dox, the Nrf2 gene expression was upregulated compared to the control (2.35 fold change). Then, the combination treatment, Dox and MO, downregulated Nrf2 expression in the MCF-7 cells (0.31 fold change) (Figure 4.8.2).

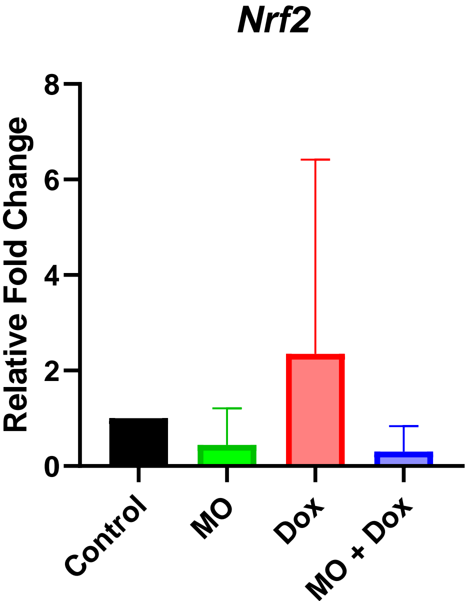

Figure 4.8.2: The effect of MO and Dox on the *Nrf2* mRNA expression in MCF-7 cells. *Nrf2* mRNA expression was used to assess oxidative damage in cells exposed to a control, Dox (0.978 µM) and MO (2600 µg/ml), as well as cells that were co-treated with MO (2600 µg/ml) + Dox (0.978 µM) for a period of 72 hours (n = 3).

CHAPTER 5: DISCUSSION

5.1 Introduction

This chapter discusses the results outlined in Chapter 4, followed by the study limitations, conclusion, as well as a summary of the study.

5.2 Antiproliferative potential of MO and Dox

Cancer has been a global burden over the years (Ma and Yu, 2006). However, the introduction of various treatment therapies, which include radiotherapy, surgery and chemotherapy, has become beneficial in the mortality and morbidity rates of cancer patients (Debela et al. 2021). Doxorubicin (Dox) continues to be the most actively and widely utilised chemotherapeutic agent in treating both early and advanced breast cancer (Lovitt, Shelper, and Avery 2018; Micallef and Baron, 2020). It has played a role in reducing cancer patient deaths worldwide (Trachtenberg et al. 2011a; McGowan et al. 2017b). However, numerous studies have reported that administering Dox results in adverse side effects that include chemoresistance and cardiomyopathy, which limits the utilisation of Dox as an effective chemotherapeutic agent (Lovitt, Shelper, and Avery 2018; Sangweni et al. 2020). Studies showed that Dox's signalling pathway interaction promotes resistance and cardiotoxicity by increasing the levels of oxidative stress whilst decreasing the activity of antioxidants and cell death (Conklin, 2005; Carvalho et al. 2009; Shabalala et al. 2017; Christowitz et al. 2019; Micallef and Baron, 2020). Previous studies revealed that post-translational modifications identified on specific proteins influence the chemoresistance mechanism of action inferred by Dox (Li et al. 2005; Shukla et al. 2010; Lovitt, Shelper, and Avery 2018; Christowitz et al. 2019; Micallef and Baron, 2020). Although Dox has been extensively investigated, its use continues to be limited as it results in drug resistance and cardiac impairment following long-term chronic exposure (Micallef and Baron, 2020; Johnson-Arbor and Dubey, 2023). Therefore, developing new and effective cancer therapeutic agents is crucial (Sangweni et al. 2020).

MO, a traditional plant that is widely consumed, is utilised as a treatment for various illnesses, including cancer (Tiloke et al. 2019; Fuel et al. 2021; Xie et al. 2020; Zunica et al. 2021). Cancer is the first leading cause of death globally, with breast cancer being the most common type (Institute 2021a; Siegel et al. 2022; Kashyap et al. 2022). This study is the first

to investigate the antiproliferative potential of MO aqueous leaf extract on the MCF-7 breast cancer cell line.

In order to determine the most therapeutic dose of MO that is effective, an MTT assay was conducted to quantify cell viability (Figure 4.1). The viability of the breast cancer cells was determined by how toxic the chemotherapeutic agent was when administered to the cells. The designated dose was used to ensure growth inhibition of the MCF-7 breast cancer cells. The findings of our study showed a decrease in cell viability of the MCF-7 cells at a concentration range of 0-5 mg/ml MO for 24, 48 and 72 hours of treatment. A 2600 µg/ml MO concentration was determined and utilised in all subsequent experiments for 72 hours. It most effectively reduced the live cells to below 50% (IC_{50}). Subsequently, MO decreased the amount of viable MCF-7 cells. This suggests that the MO aqueous leaf extract was cytotoxic to the MCF-7 cells at a prolonged exposure concentration of 2600 µg/ml. The cytotoxicity findings of this study were comparable with a study conducted by Adebayo and colleagues (2017; 2018) as well as Elsayed et al. (2015), who showed an inhibition of MCF-7 breast cancer cell proliferation; however, using crude extracts and fractions of the MO seeds. Virk and co-workers (2023) also demonstrated similar findings by showcasing MO's cellular inhibitory ability in both MCF-7 and $HepG_2$ cells (Virk et al. 2023). The introduction of Dox has previously been shown to decrease the viability of MCF-7 breast cancer cells (Pilco-Ferreto and Calaf, 2016). Similarly, it was observed that the administration of Dox to the MCF-7 breast cancer cells suppressed cell proliferation in a dose-dependent manner over 72 hours with an IC_{50} concentration of 0.978 µM (Figure 4.2). These findings were supported by a study conducted by Sangweni and colleagues (2020), who showed a decrease in viability of MCF-7 cells following Dox treatment (Sangweni et al. 2020). These results were further supported by another study which demonstrated a decrease in cell viability, however, in a different cell line, namely hepatocellular carcinoma, through the signalling pathway that involves the PTP4A3 expression being downregulated via IL-R6-JAK2-STAT3 (Li et al. 2023).

The molecular variations, as well as disturbances of the homeostasis of the glutathione antioxidant system, have been associated with the initiation and progression of cancer (Kennedy et al. 2020). In healthy cells, carcinogens must be removed and detoxified (Kennedy et al. 2020). However, in cancerous cells, increased levels of GSH are responsible for the rising progression and increased resistance to chemotherapeutic drugs, which are side effects inferred by Dox (Traverso et al. 2013; Christowitz et al. 2019; Kennedy et al. 2020;

Levi et al. 2021). This study's findings showed that the introduction of Dox resulted in increased ROS production (Figure 4.4), which has been previously shown to be responsible for its antiproliferative potential in cancer cells (Traverso et al. 2013; Gopalakrishnan, Doriya, and Kumar 2016; Christowitz et al. 2019; Kennedy et al. 2020; Levi et al. 2021).

The leaf extract of MO has been found to contain several phytochemicals, including gallic acid, catechol and itaconic acid, which are flavonoids. The aqueous leaf extract of MO is mainly composed of hydrocarbons and phenolic acids (Tiloke, 2015). The chemical composition of these compounds was illustrated by Tiloke (2015), utilising the Gas Chromatography/Mass Spectrometry (GC-MS) analysis. Moreover, the high levels of phenolic acids found in MO were shown to be accountable for their antioxidant potential (Kashyap et al. 2022). One of the most potent antioxidants found in MO leaf is benzyl isothiocyanate, which is responsible for the anticancer characteristic by increasing intracellular ROS and cell death. It was discovered that the administration of MO to the MCF-7 breast cancer cells led to a significant decrease in GSH production (Figure 4.3) whilst increasing ROS (Figure 4.4). This study's findings were supported by a study conducted by Tiloke and colleagues, which also showed a decrease in GSH following MO administration and induced oxidative stress in lung and liver cancer cells (Tiloke et al. 2013; 2019). This study suggests that the bioactive compounds including benzyl isothiocyanate play an important role in reducing the proliferation of MCF-7 cells exposed to MO by resulting in oxidative stress (Lee et al. 2017). This study also showed that the combination of MO and Dox decreased the production of GSH, which demonstrated the induction of oxidative stress in the MCF-7 breast cancer cells (Figure 4.3). Elevated ROS production was also seen in the co-treatment of MO and Dox in the MCF-7 breast cancer cells (Figure 4.4).

The induction of ROS in cancer cells has been indicated to cause apoptosis (Brentnall et al. 2013; Fourie et al. 2019). Apoptosis is executed by caspases, which act as primary mediators of the cell death pathway. This includes the effector (caspase-3/7) and initiator caspases (caspase-8 and -9). Caspase-9 activates caspase-3/7, leading to cell death (Green and Llambi, 2015). Recently, it was discovered that MCF-7 breast cancer cells lack caspase 3. It results from a 47- base pair deletion mutation in the gene's exon 3. However, the overexpression of caspase 3 in MCF-7 cells suggests that caspase 3 is essential for the apoptotic pathways mediated by mitochondria and death receptors (Yang et al. 2001; Tian, 2023). This study demonstrated that MO significantly increased the activity of caspase-3/7 (Figure 4.5.1.3) as well as -8 (Figure 4.5.1.2) and -9 (Figure 4.5.1.1), causing apoptosis in the MCF-7 breast

cancer cells. The same response was observed in cells exposed to Dox and the combination treatment of MO and Dox, where there was increased cell death. However, the combination treatment had the greatest effect on both initiator and effector caspase activity. The results in this study were supported by Fourie and colleagues (2019), who demonstrated that the cleavage of caspase-3 and -8 increased apoptosis significantly in Dox-treated cells (Fourie et al. 2019). Therefore, This study's results demonstrate that MO and Dox induce apoptosis in the MCF-7 cells, a crucial aspect of cancer therapy.

Cell death has various mechanisms unique in their signalling cascades, and ATP plays a role in mediating all types of apoptosis (Zhou et al. 2012; Imamura et al. 2020). Cancer has high metabolic activity and an elevated energy demand (Tiloke, 2015; DeBerardinis and Chandel, 2016; Bosque et al. 2023). Increased metabolic activity enhances ROS levels, resulting in the induction of oxidative damage (Tiloke, 2015; DeBerardinis and Chandel, 2016). This study found that both MO as a mono treatment and the combination of MO and Dox significantly increased the metabolic activity of the cells, thus leading to cell death (Figure 4.6). Our results suggested that when levels of ATP are high in MO-treated cells, increased amounts of ROS are produced (Figure 4.4), whilst GSH levels decrease (Figure 4.3), which induces cell death. A previous study supported this study's findings and revealed that the aqueous extract of moringa stenopetala leaves raised the amount of ATP at a high dosage (Kerai and Timbrell, 1997; Abd-Rabou et al. 2017). The authors indicated that the presence of carbohydrates at high levels in the extract could explain the rise in metabolic activity (Kerai and Timbrell 1997; Abd-Rabou et al. 2017). The presence of ATP led to the conformational change in the adapter protein called the apoptotic protease-activating factor 1 (Apaf-1) (Wurstle and Rehm, 2014). This happens when there is mitochondrial polymerisation, which gives rise to the release of cytochrome c (Molatlhegi et al. 2015; Würstle and Rehm 2014). The cytochrome c works with Apaf-1 to form the apoptosome complex (heptameric backbone), which activates caspase-9 (Figure 4.5.1.1). Caspase-9 cleaves and subsequently leads to the activation of caspase-3/7 (Figure 4.5.1.3), which results in cell death (Molatlhegi et al. 2015). A contradiction to our results was outlined by a previous study conducted by Imamura et al. (2020), who mentioned that the level of ATP declines exactly after caspase-3 is activated for cell death to occur. However, for the progression of apoptosis to take place, a sufficient amount of ATP is needed (Imamura et al. 2020). The results found by Madi and colleagues (2016) also contradicted this study's findings by demonstrating that MO leaf extract reduced the levels of ATP, reducing the concentration of live cells (Madi et al. 2016).

The current findings showed that MO can be a possible chemotherapeutic regimen as it displayed antiproliferative potential in the MCF-7 cells. However, more investigations still need to be conducted including animal studies to validate our results. Additionally, in contrast to cell death, previous studies have indicated that high levels of ATP may be involved in the induction of chemoresistance and cell proliferation, which can be a future topic to be investigated further (Chen and Yu, 2020; Fiorillo et al. 2021). It was observed that Dox, as a mono treatment, exhibited a moderate decrease in ATP levels when it was compared to the control cells (Figure 4.6). This study's results were supported by a study conducted by Sangweni and colleagues (2023), who showed a reduction in the metabolic activity of MCF-7 breast cancer cells following Dox exposure (Sangweni et al. 2023). This suggested that Dox could influence the process by inducing oxidative damage, which depolarised the mitochondrial membrane, thus inducing cell death. This study also showed that Dox increased GSH levels in the MCF-7 cells following 72 hour exposure. Previous studies have showed that elevated levels of GSH induces chemoresistance (Traverso et al. 2013; Christowitz et al. 2019; Kennedy et al. 2020; Levi et al. 2021). Therefore, since there are bioactive compounds that have been shown before to play a role in the antiproliferative potential MO has in cancer, we further want to investigate which ones are there in the aqueous leaf extract Similarly, the combination treatment MO + Dox displayed increased levels of GSH though lower than the mono-treatment of Dox. These findings may suggest that MO played a role in decreasing the effects inferred by Dox in the MCF-7 cells.

The mRNA is important for cell protein production (Brown and Clancy, 2008). The Nrf2 signalling axis aims to target the bioactive native electrophiles and covalent drugs (Poganik et al. 2019). However, it is understood that the regulation of Nrf2 is focused at the protein level (Poganik et al. 2019). Nrf2 is pivotal in defending the cell against oxidative damage (Tiloke, Phulukdaree, and Chuturgoon 2013). The Nrf2 protein inhibits ROS and promotes oxidative damage repair through the production of its target genes, hindering the process of carcinogenesis (Zhang et al. 2015). One of the most predominant pathways in the anti-tumorigenesis mechanism is the Nrf2/Keap1 (Wu et al. 2019). Nrf2 separates from the Kelch-like epichlorohydrin-associated protein 1 (Keap1), therefore translocating to the nucleus. This results in Nrf2 binding to the antioxidant response elements in regions that promote the antioxidant genes, increasing the transcription process (Tiloke, Phulukdaree, and Chuturgoon 2013). The preliminary protein expression of Nrf2 in MO-treated cells was suppressed by 0.614 fold change (Figure 4.7.1). This resulted in oxidative stress in the cells by increasing

ROS levels (Figure 4.4) whilst decreasing the transcription of key antioxidant genes, in this case, GSH (Figure 4.3) (Tiloke, Phulukdaree, and Chuturgoon 2013). The protein Nrf2 has a dual role. It can inhibit or progress cancer development. However, the evident downregulation of Nrf2 may be a reason for the antiproliferative effect seen in the MCF-7 cells following exposure to MO (Figure 4.7.1). This study's findings are supported by a study conducted by Tiloke et al. (2013), which showed that Nrf2 at low levels results in oxidative stress, thus decreasing the proliferative potential of cancerous cells. The above western blot preliminary findings may mean that MO induced Keap-1 activity, therefore targeting the Nrf2 protein for ubiquitin-mediated degradation. Nrf2 is also responsible for regulating GSH synthesis (Tiloke 2015; Kitamura and Motohashi 2018). The downregulation of Nrf2 in the presence of MO was in correlation with the levels of GSH in this study (Figure 4.3). Moreover, our preliminary results also showed that *Nrf2* mRNA expression was reduced in MO-exposed cells by 0.44 fold change compared to the control (Figure 4.8.2), which was in line with the low Nrf2 protein levels. These results corresponded with a study by Tiloke (2015) demonstrating low *Nrf2* mRNA expression in MO-treated cells. A similar result was observed at the protein level in the Dox-treated cells, which showed low Nrf2 protein levels in the MCF-7 cells (0.258 fold change) as well as in the cells that were exposed to the co-treatment of MO and Dox when compared to the control (0.788 fold change). Additionally, at the gene level, there was a decrease in *Nrf2* mRNA expression by 0.31 fold change in the MO + Dox treated cells (Figure 4.8.2)., which corresponded with a decrease in expression at the protein level, relative to control (Figure 4.7.1). However, increased expression of *Nrf2* mRNA was observed in Dox-treated cells (2.35 fold change) (Figure 4.8.2). Overexpression of Nrf2 has been shown to induce drug resistance, inhibiting the efficacy of cancer treatment (Zhang et al. 2015; Kumar et al. 2022). Therefore, this study's results suggest that changes following Dox treatment may have contributed to the overexpression of the *Nrf2* gene in the MCF-7 cells. Inhibiting the activation of Nrf2 can be regarded as an ideal strategy to reverse the resistance inferred by the protein. In addition, the Nrf2 protein activates the expression of an apoptotic protein known as p53, a protein linked to cell death (Cirone and D'Orazi 2023).

The tumour suppressor gene, p53, has the function of inhibiting and eliminating the metastasis of abnormal cells (Gasco et al. 2002). This gene's signalling is activated when stressors are introduced, such as DNA damage (Gasco et al. 2002). The levels of p53 in unstressed cells are low; this was evident in the control cells of this study (Figure 4.7.2). The

protein is targeted for proteasomal degradation via the E3 ubiquitin ligase Mdm2. In cells that are not transformed, the induction of cell death by p53 is usually through direct activation of transcription of the p53 PUMA, which are the pro-apoptotic BH3-only proteins as well as NOXA. PUMA and NOXA are known as the effectors of cell death in the p53 pathway. The loss of both cell cycle arrest (p21) and the p53 apoptotic effectors prohibits aggressive tumour growth. The stimulation of the cell through NOXA and PUMA is imperative for the elimination of cancerous cells. The removal of these malignant cells is done by anti-tumour drugs, which initiate TP53 even though additional effectors play a role (Gasco et al. 2002). Increased p53 levels encourage biological processes, including apoptosis, a well-known tumour-suppressing mechanism that prevents uncontrolled cancer cell division or eradicates them entirely (Meek 2015). The preliminary western blot results following 72-hour exposure showed that MO could upregulate the protein levels of p53 (Figure 4.7.2) compared to the untreated cells (4.345 fold change). This increased p53 MO-induced apoptosis following severe DNA damage might be through the activation of pro-apoptotic BH3-only proteins as well as PUMA. This study's results were supported by a study conducted by Cirmi and colleagues (2019), who showed elevated p53 protein levels in MO-treated human neuroblastoma cells (Cirmi et al. 2019). Also, this study's results were further validated by Tiloke (2015), however, in lung cancer cells (Tiloke 2015). The p53-mediated pathway has been shown to respond to cellular stresses and induce cell death (Abd Karim et al. 2023). The highly expressed p53 protein in this study accounts for the upregulation of caspase-3 (Figure 4.5.1.3) whilst downregulating the expression of Nrf2 (Figure 4.7.1) as well as Bcl-2 (Figure 4.7.3) (Abd Karim et al. 2023).

Moreover, there was a slight increase in the expression of p53 in Dox-treated cells (1.065 fold change) (Figure 4.7.2), as also found by Lin and co-workers (2018), who demonstrated high levels of p53 protein in Dox-treated prostate cancer cells. The authors suggested that severe DNA damage is the reason for elevated Dox-induced p53 apoptosis (Lin et al. 2018). The co-treatment of MO + Dox also showed a modest upregulation of p53 protein levels in the MCF-7 cells compared to the control (1.026 fold change), which also suggests severe damage to DNA. When DNA damage is too extensive to repair, p53 triggers apoptosis in the cell. Nearly every cell stress response, including gene expression, DNA repair, cell cycle arrest, metabolic changes, apoptosis, and senescence, appears mediated by the p53 pathway (Elkholi and Chipuk, 2014). Furthermore, p53 leads to the induction of multiple target gene expression such as $p21^{CDKNIA}$ as well as mouse double minute 2 homolog (Mdm2) (López et

al. 2015). These genes determine whether the cell will undergo apoptosis, senescence or DNA repair processes (López et al. 2015). In this study, MO downregulated the gene expression of p53 by 0.71 fold change compared to the control. The p53 gene has been shown to have various mechanisms of action. These include its potential to regulate mutation at the genetic level, lead to the inhibition of epigenetic p53 transcription at the transcriptional level, induce protein folding, and p53 posttranslational modifications (Liu, Tavana and Gu, 2019). These modifications include phosphorylation, acetylation, and methylation, to name a few. Liu and colleagues (2019) stated that the loss of function of p53 makes the cells susceptible to continuous damage as well as neoplastic transformation in mouse experiments (Liu, Tavana and Gu, 2019). The authors also mentioned that ataxia-telangiectasia mutant kinase and checkpoint kinase 1/2 (Chk1/2) can phosphorylate S15 (mouse S18) and S20 (mouse S23), respectively (Liu, Tavana and Gu, 2019). These regions are phosphorylated to shield p53 from its key negative regulator, Mdm2, enhancing stability and function. Furthermore, Ser-Thr kinase VRK1 has the ability to phosphorylate p53 at T18 once cells have been subjected to various forms of DNA damage (Liu, Tavana and Gu, 2019).

The Deubiquitinases can remove ubiquitin from p53. A crucial p53 regulator known as herpesvirus-associated ubiquitin-specific protease (HAUSP, also known as USP7) can deubiquitinate p53 (Liu, Tavana and Gu, 2019). Even in the presence of excessive Mdm2, HAUSP can stabilise p53 *in vitro* and *in vivo*. Interestingly, while the entire loss of HAUSP stabilises and activates p53, moderate reduction of endogenous HAUSP levels destabilises endogenous p53. Additional research has shown that HAUSP can also deubiquitinate Mdm2. When HAUSP is suppressed, Mdm2 will become quite unstable due to its autoubiquitination, then stabilising p53 (Liu, Tavana and Gu, 2019).

Deubiquitinating enzymes that control the stability of p53 E3 ligases can also be targeted to prevent Mdm2 and Mdmx from acting on p53. By destabilising both Mdm2 and Mdmx, for instance, the authors have shown that inhibiting HAUSP indirectly stabilises the levels of the p53 protein (Liu, Tavana and Gu, 2019). Inhibitors that target the enzymatic activity of HAUSP have been created as a result of these important investigations (Liu, Tavana and Gu, 2019). Two HAUSP inhibitors, P22077 and P5091, were identified by high-throughput screening and were shown to stop the growth of neuroblastoma or multiple myeloma *in vivo*, respectively. It is interesting to note that p53 has Mdm2 as a transcriptional target. Therefore,

a double-negative regulatory loop can be formed by p53 and Mdm2 (Liu, Tavana and Gu, 2019).

In order to trigger p53-mediated apoptosis, S46 at p53 will also be phosphorylated if the DNA damage stress is severe enough. However, genes that cause cell cycle arrest will not be activated such as p53-regulated apoptosis-inducing protein 1 (p53AIP1). This suggests how p53 alteration affects the p53 target selectivity (Liu, Tavana and Gu, 2019). The ability of p53 to increase function and be stable in both the nucleus and cytoplasm to control the cellular phenotype after stress is enabled by transcriptional activation in conjunction with post-translational changes of the p53 protein (Elkholi and Chipuk, 2014). Therefore, this study suggests that p53 may be able to induce apoptosis indirectly via different modification mechanisms, including phosphorylation as well as acetylation. Similar results were seen in Dox-treated cells and the combination treatment whereby p53 gene expression was decreased by 0.87 fold change and 0.65 fold change compared to the control, respectively (Figure 4.8.1). The p53-mediated apoptosis depends on p53 localisation to the mitochondria, which influences the production of certain proteins, such as the Bcl-2 family of proteins. These proteins thus regulate the permeability of the power cell organelle, mitochondria (Bai and Zhu, 2006).

Bcl-2 family members have been categorised based on their domains and functions (Qian et al. 2022). Bcl-2 family proteins are divided into categories, including anti-apoptotic, also referred to as pro-survival proteins inhibiting cell death and pro-apoptotic proteins, which induce apoptosis, respectively (Qian et al. 2022). An anti-apoptotic protein, Bcl-2 is a regulator of cell death and many other functions (Hardwick and Soane, 2013). It plays a role in mediating and controlling how the intrinsic apoptotic pathway in the power cell, the mitochondria, promotes intrinsic apoptosis (Hardwick and Soane, 2013). It contributes to the prevention of apoptosis in cancer, allowing cancer cells to survive under adverse conditions (Adams and Cory 2018). This anti-apoptotic member, Bcl-2, is known to neutralise the function of Bax to initiate apoptosis (Sharifi et al. 2014). The intrinsic pathway of cell death is pivotal for normal fetal development as well as cancer prevention (Hardwick and Soane, 2013). It has been reported previously that Bcl-2 proteins are overexpressed in various cancers, including breast cancer, and the overexpression enhances the capability of these cancer cells to migrate and invade (Um, 2016; Lucantoni et al. 2021). However, this study showed that MO could be a possible anti-cancer drug as it downregulated the expression of

Bcl-2 protein (0.205 fold change) (Figure 4.7.3). This was supported by a study conducted by Hagoel et al. (2018), Do et al. (2021) as well as Kumar and colleagues (2023), but for different cell lines. In this study, MO induced pro-apoptotic signals, which prevented cancer cell survival following stressful conditions. Furthermore, when cells were treated with Dox, this study showed that Bcl-2 decreased with a 0.047 fold change compared to the control (Figure 4.7.3). The inhibition was also seen in a previous study conducted by Vu et al. (2020), who showed similar results where Dox downregulated the expression of Bcl-2, however, in acute myeloid leukaemia. When the cells were exposed to MO and Dox co-treatment, the Bcl-2 levels were also low compared to the control (0.099 fold change) (Figure 4.7.3). Therefore, this study suggests that MO could be a possible antiproliferative agent as it prevented the Bcl-2 protein from inhibiting apoptosis.

Bax is a Bcl-2 homolog that resides primarily in the cytosol. It is considered an essential effector of intrinsic (mitochondrial) cell death induced by cancer therapeutics (Lopez et al. 2022). It is a member of the pro-apoptotic Bcl-2 gene family, and in the presence of stress in the cells, p53 leads to the activation of Bax. This results in oligomerisation, the formation of pores in the outer membrane of the mitochondria, thus releasing cytochrome c and further inducing cell death (Chen and Overholtzer, 2018). Bax has been shown to maintain respiration in non-stressful conditions or the proliferative capability, migration, and differentiation of unstressed cells, however, in the nucleus (Chen and Overholtzer, 2018). Bax is necessary for inciting apoptosis by triggering the permeabilisation of the mitochondrial membrane (Adams and Cory 2018).

Moreover, protein Bax is induced by p53. Elevated expression of Bax is known to increase sensitivity to apoptosis stimuli and decrease cancer cell growth (Manoochehri et al. 2014; Sharifi et al. 2014)—however, reduced expression of Bax results in the inhibition of pathways involved in cell death. This study found that exposure to MO in the MCF-7 cells downregulated the expression of Bax (0.317 fold change) compared to the control (Figure 4.7.4). These results suggest that low expression of Bax might be associated with resistance in the MCF-7 cells. These results were validated by a previous study that showed that MO was able to decrease the expression of Bax, however, in renal tubular cells (Jannah et al. 2018). The authors suggested that it may be because antioxidants contained in MO lead to the inhibition of ROS when there is paracetamol induction, thus preventing cell damage (Jannah et al. 2018). However, cancerous cells tend to proliferate indefinitely at a high energy cost, necessitating a continuous flow of ATP (Boohaker et al. 2011). According to the Warburg

effect, malignant cells produce energy through glycolysis rather than oxidative phosphorylation. Boohaker and colleagues mentioned that it is likely that the glycolytic pathway could have been favoured by a partial loss of Bax, which is seen in many malignancies (Boohaker et al. 2011). This suggests that partial activation of Bax would be more likely to support the development and metabolic requirements of cancer cells than total inactivation (Boohaker et al. 2011). Therefore, the downregulation of p53 mRNA expression (Figure 4.8.1) may account for the partial activation of Bax protein levels in MO-treated cells (Figure 4.7.4) rather than highly increased levels of Bax when compared to the control.

A similar effect was observed when cells were treated with Dox, displaying a 0.114 fold change decrease of Bax in the MCF-7 cells (Figure 4.7.4). Similarly, cells exposed to the co-treatment of MO + Dox showed downregulation of Bax protein expression compared to the control (0.097 fold change) (Figure 4.7.4). Loss of Bax has been previously shown to be associated with resistance in Burkitt lymphoma cells (Ierano et al. 2013). The authors believe this may be due to post-transcriptional effects, though they do not show the data. Moreover, a previous study conducted by Krajewski and colleagues (1995) suggested that lower expressions of Bax in cancer indicate poor response to chemotherapeutic regimens as well as decreased survival rates in women with metastatic breast cancer. These results were further supported by Pluta et al. (2011), who suggested that low expressions of Bax in breast cancer could be one of the mechanisms which cancer cells utilise to avoid cell death.

PARP-1 is considered the primary component for the functioning of several cellular processes such as transcription regulation, response to DNA damage, and cell death (Rose et al. 2020). Amid cell death, there is a cleavage of PARP-1, and its role is to detect and repair single-strand DNA breaks (Tiloke, Phulukdaree, and Chuturgoon 2013; Martí et al. 2020; Rose et al. 2020). The inhibitors of PARP-1 have become an important aspect of cancer therapy as they block the enzymes involved in the repair of DNA (Matulonis and Monk, 2017; Li et al. 2020; Kim and Nam, 2022). This study revealed that MO inhibited the protein levels of PARP-1 in MCF-7 treated cells (0.053 fold change) (Figure 4.7.5). This meant that MO had the potential to inhibit DNA repair. This study’s results were supported by a study investigated by Hagoel and co-workers, who showed lower levels of PARP-1 following MO treatment of pancreatic cancer cells (Hagoel et al.2019; Rose et al. 2020). The authors highlighted that the downregulation of PARP-1 led to the delay of DNA repair (Hagoel et al. 2019).

Moreover, when the MCF-7 cells were exposed to Dox, there was a decrease in the expression of the PARP-1 enzyme (0.031 fold change) (Figure 4.7.5). These results were supported by Wang and colleagues (2012), who showed that Dox inhibited pre-implantation development in early mouse embryos by inactivating PARP-1 DNA repair (Wang et al. 2012). Additionally, the combination of MO + Dox showed similar results of lower PARP-1 levels in the MCF-7 cells (0.472 fold change). The above results from this study provided evidence that MO possess cytotoxic effects which induced both oxidative stress and apoptosis in MCF-7 cells. Thus, demonstrating that the aqueous leaf extract of MO may be a possible antiproliferative agent for the MCF-7 breast cancers.

CHAPTER 6: CONCLUSION

This chapter concludes the study following all analyses of the results and outlines the recommendations and limitations of the study.

This study displayed that Dox, the currently approved chemotherapeutic drug, has anti-cancer properties against cancer. Dox decreased the cell viability of the breast cancer cells and increased the levels of ROS. The introduction of Dox to the cells also induced apoptosis through the activation of caspases, thus leading to DNA damage. However, antioxidants and other apoptotic proteins were suppressed, resulting in resistance. These results, therefore, support the notion that Dox induces chemoresistance in cancer cells, as proven by previous studies.

Since Dox has been proven to induce resistance in cancer cells, alternative treatments such as plant-based therapy (e.g., MO) are of interest. This study showed that MO's anticancer capabilities result from its ability to cause p53-mediated apoptosis in breast cancer cells by inducing oxidative stress and suppressing the production of antioxidant defence proteins and anti-apoptotic proteins. DNA damage was the outcome, and tumour suppressor genes, pro-apoptotic proteins, and apoptosis executioner molecules were then upregulated. Even though proteins such as Bax were downregulated for apoptosis, most of the apoptotic proteins investigated induced cell death, thus counteracting the negative feedback actioned by Bax (Figure 4.7.4). These outcomes of MO have been proven by previous studies and our study confirms them.

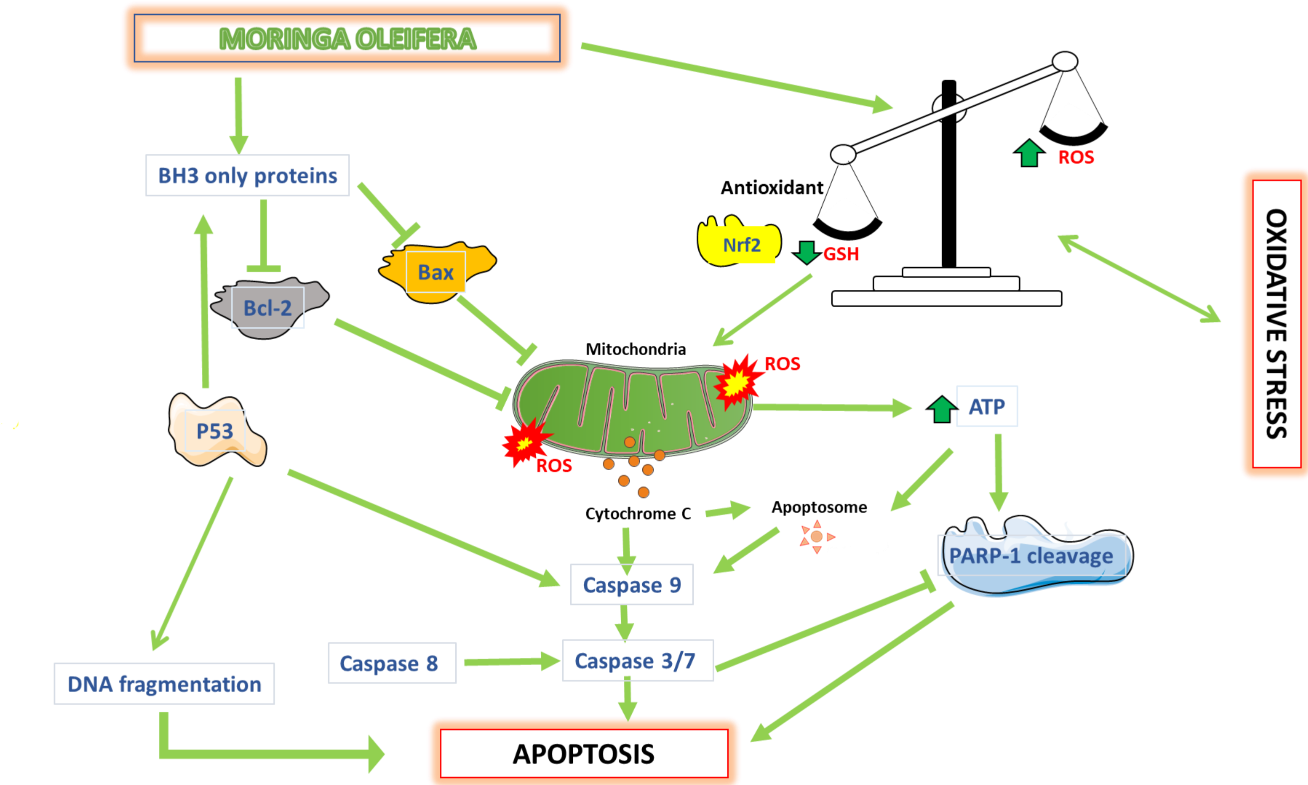

Figure 6: The effect of MO on the MCF-7 breast cancer cells. MO induced oxidative stress in breast cancer cells, which suppressed the synthesis of antioxidant defence proteins and anti-apoptotic proteins, leading to p53-mediated death. The DNA fragmentation consequently increased tumour suppressor genes, pro-apoptotic proteins, and apoptosis executioner molecules.

The study outcomes also showed a possible synergistic anticancer potential of MO and Dox combination in breast cancer. This was observed with the induction of oxidative stress and reduced cell viability of the breast cancer cells. Moreover, the activation of cell death executioner molecules, tumour suppressor genes, and pro-apoptotic proteins resulted. This caused DNA damage and further led to cell death. Thus, MO can be a suitable candidate against breast cancer proliferation by inducing oxidative damage and apoptosis in the MCF-7 breast cancer cells. Considering that this was an *in vitro* study, more research is required to fully describe this novel compound, its proposed mechanism of action, and assess its specificity. Comet assay can be conducted to assess DNA damage. Different leaf extracts have been investigated before, however, our study is the first to investigate the aqueous leaf extract on the MCF-7 cells. Since there are bioactive compounds that have been shown before to play a role in the antiproliferative potential MO has in cancer, we further want to investigate which ones are there in the aqueous leaf extract. The high-performance liquid

chromatography with tandem mass spectrometry may be utilised to identify and separate bioactive compounds. Furthemore, since we used Dox as a positive control in our study, future studies may entail us looking at the drug to drug interaction between MO + Dox to confirm their reaction with each other when co-administered. *In vitro* models play a role of demonstrating whether a drug is effective or not through a series of experiments. Once it demonstrates effectiveness, further investigations can be conducted using *in vivo* models which advance drug development studies. Both models are important in evaluating the efficacy of candidate drugs. Therefore, advanced cell-based models such as spheroids and organoids (3D cell culture), as well as animal models for *in vivo* studies as they mimic the natural environment of cells, can also be used to help further characterise this novel drug after it has been properly characterised *in vitro* to better understand its mode of action. Precision medicine such as theranostics aids in helping physicians determine those who may be more susceptible to cancer and assist them in reducing their specific disease risk. Providing the appropriate cancer treatment to the right patient utilising the correct dose and timing is the main objective of precision medicine. Our study revealed the effective dose and time frame at which MO leaf extract was effective. Next generation sequencing may be utilised in future studies for targeted therapy and in helping to assess specific patient disease risks.

6.1. Limitations

A limitation of the study included that for western blot, it was not possible to conduct repeat experiments to make an n=3; thus, no significance in the results was obtained. This was due to the delay in reagent deliveries from the supplier. Increasing the number of replicates may allow for a narrower margin error and improve the precision of experiments.

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APPENDICES

Appendix A

Dose response curves for 24 and 48 hours

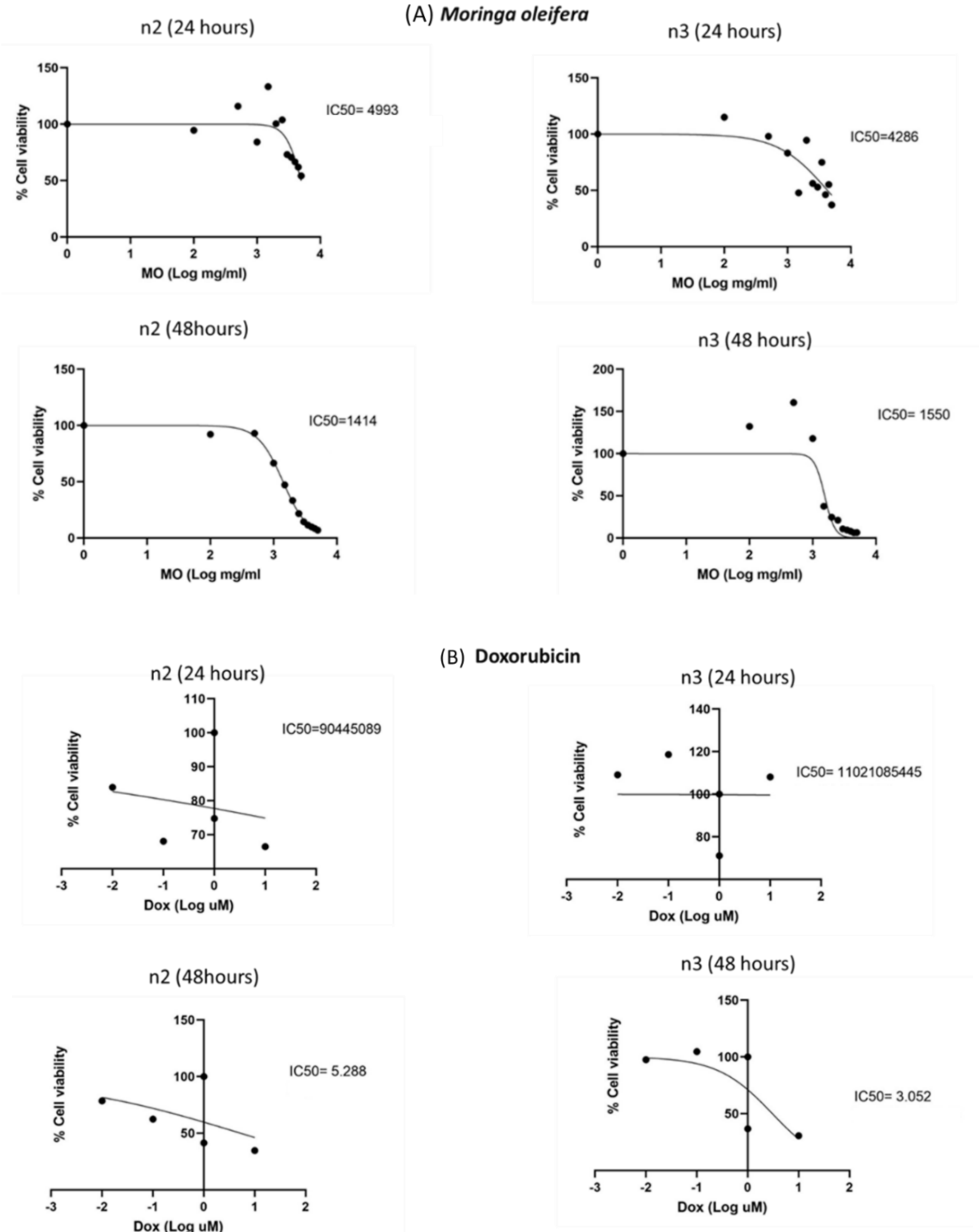


Figure 1: (A) The IC_{50} concentrations of MO following 24 and 48 hours treatment exposure . **(B)** The IC_{50} concentrations of Dox after 24 and 48 hours treatment. N2 and N3 represents the number of repeats.

Appendix B

Moringa oleifera plant Identification hebarium letter

KwaZulu-Natal Herbarium (NH)
South African National Biodiversity Institute

P.O. Box 52099, Berea Road, 4007
South Africa
Tel: +27 31 2024095, Fax: +27 31 2023430
Email: KwaZulu-NatalHerbarium@sanbi.org.za

Ref: Plant Identification Dispatch List 19 January 2022

Client: Dr Charlette Tiloke
Address: Staff No. 0890138
University of the Free State
Department of Basic Medical Science

Tel:
Cell: 0845251340
Email: Tilokec@ufs.ac.za

ID CODES:
0 = No challenges
1 = Specimen too poor to ID
2 = Label information inadequate
3 = Cannot match specimen in herbarium
4 = Specialist not available to do ID
5 = Genus requiring/under revision
6 = Specimen closest to name listed (cf)
7 = Please send more material
8 = Please refer to attached note/letter
9 = New record

FATE:
K = specimen kept for herbarium
R = specimen returned
S = specimen scrapped

	Collector	No.	Plant Name	Det. By	Det. Notes	ID Code	Fate
1	C. Tiloke	2	*Moringa oleifera Lam.*	Ngwenya, A.M. 19/1/2022			

Curator
KwaZulu-Natal Herbarium (NH)

Please note a handling fee is charged for each specimen received for identification. 1

Appendix C

Ethics letter (Initial and extension approval)

Health Sciences Research Ethics Committee

08-Dec-2021

Dear **Ms Malebogo Moremane**

Ethics Clearance: **Investigating the potential antiproliferative effect of Moringa oleifera aqueous leaf extract in MCF-7 breast cancer cells**

Principal Investigator: **Ms Malebogo Moremane**

Department: **Basic Medical Sciences Department (Bloemfontein Campus)**

Submission Page

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2021/1631/2501**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

Research conducted in any Department of Health facility: Researchers are required to sign and return the HSREC approval letters to the provincial Department of Health where they applied. It is also a requirement for researchers to submit electronic copies of their final research findings, and/or make a presentation of their findings and recommendations at departmental research days when and where indicated.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

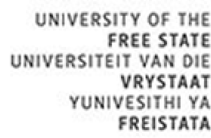
For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Prof. A. Sherriff
Chairperson: Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za



Health Sciences Research Ethics Committee

21-Nov-2022

Dear **Ms Malebogo Moremane**

Ethics Number: UFS-HSD2021/1631-0005

Ethics Clearance: **Investigating the potential antiproliferative effect of Moringa oleifera aqueous leaf extract in MCF-7 breast cancer cells**

Principal Investigator: **Ms Malebogo Moremane**

Department: **Basic Medical Sciences Department (Bloemfontein Campus)**

Submission Page

SUBSEQUENT SUBMISSION APPROVED

Your recent submission for ethical clearance with the Faculty of Health Sciences has been approved by the Health Sciences Research Ethics Committee (HSREC). We acknowledge receipt of the following documents:

The ethics clearance of this project is extended until 20 November 2023.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2020); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

The Principal Investigator (PI) bears final responsibility for the RIMS application. In the event of any misconduct or improper activities perpetuated by a third party, the PI will be held vicariously liable. The HSREC will bear no responsibility or liability for any actions of a PI and/or third party or breach of confidentiality caused by the PI and/or third party.

For any questions or concerns, please feel free to contact HSREC Administration.

We wish you continued success with your research.

Yours Sincerely

Prof. A. Sherriff
Chairperson : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7387 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
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Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix D

BSA linear graphs obtained for protein quantification

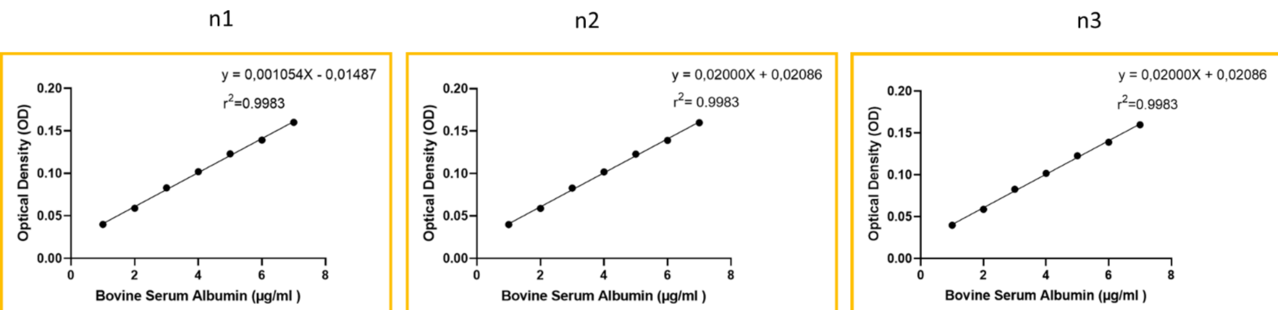

Figure 2: Standard curve utilising different known BSA concentrations for protein quantification

Appendix E

The plot graph displaying the levels of ROS in the MCF-7 cells utilising the flow cytometry technique

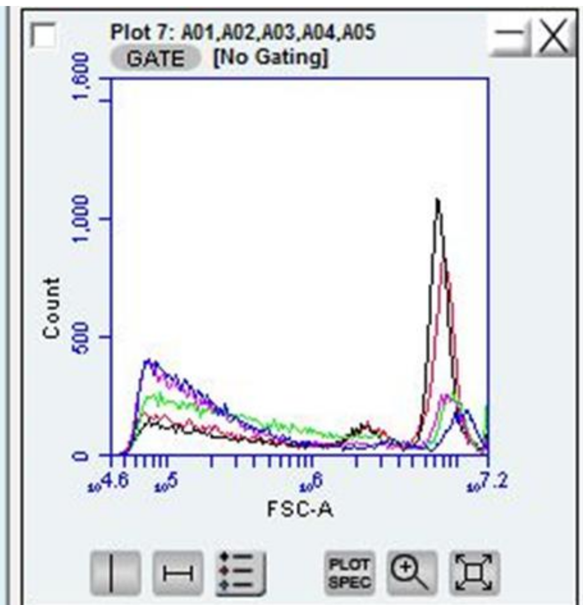

Figure 3: Graph representation of intracellular ROS levels following 72 hours' treatment exposure using the DCFH-DA assay. Colour codes: Red- Unstained control; Black- Control; Green- MO; Pink- Dox; Blue- MO + Dox

Appendix F

Certificate of publication

current issues in molecular biology

an Open Access Journal by MDPI

Indexed in: PubMed

CITESCORE 2.4

IMPACT FACTOR 3.1

CERTIFICATE OF PUBLICATION

Certificate of publication for the article titled:

Moringa oleifera: A Review on the Antiproliferative Potential in Breast Cancer Cells

Authored by:

Malebogo M. Moremane; Beynon Abrahams; Charlette Tiloke

Published in:

Curr. Issues Mol. Biol. **2023**, Volume 45, Issue 8, 6880-6902

MDPI Academic Open Access Publishing since 1996

Basel, August 2023

Appendix G

Preview of publication

Review

Moringa oleifera: A Review on the Antiproliferative Potential in Breast Cancer Cells

Malebogo M. Moremane, Beynon Abrahams and Charlette Tiloke *

Department of Basic Medical Sciences, School of Biomedical Sciences, Faculty of Health Sciences, University of the Free State, Bloemfontein 9301, South Africa; 2018305264@ufs4life.ac.za (M.M.M.); abrahamsbr@ufs.ac.za (B.A.)

* Correspondence: tilokec@ufs.ac.za

Abstract: The global burden of female breast cancer and associated deaths has become a major concern. Many chemotherapeutic agents, such as doxorubicin, have been shown to have adverse side effects. The development of multi-drug resistance is a common occurrence, contributing to chemotherapeutic failure. The resistance of breast cancer cells to drug treatment leads to a decline in the treatment efficacy and an increase in cancer recurrence. Therefore, action is required to produce alternative drug therapies, such as herbal drugs. Herbal drugs have been proven to be beneficial in treating illnesses, including cancer. This review aims to highlight the antiproliferative potential of *Moringa oleifera* (MO), a medicinal tree native to India and indigenous to Africa, in breast cancer cells. Although MO is not yet considered a commercial chemopreventive drug, previous studies have indicated that it could become a chemotherapeutic agent. The possible antiproliferative potential of MO aqueous leaf extract has been previously proven through its antioxidant potential as well as its ability to induce apoptosis. This review will provide an increased understanding of the effect that MO aqueous leaf extract could potentially have against breast cancer.

Keywords: apoptosis; breast cancer; oxidative stress; antiproliferative; *Moringa oleifera*

Appendix H
Hoescht staining assay

The Hoescht staining assay was utilised to analyse cells that are live by generally measuring the content of DNA in the cultured cells and also assessing the presence of mycoplasma in the cells. The Hoescht stain is a fluorescent nucleic dye that is permeably blue and is utilised in fluorescent microscopy. The fluorescence becomes high following binding to the adenine-thymine regions of the DNA in the minor groove. Fluorescent nuclei are visible in cells that are not contaminated whilst there are filaments as well as small cocci in mycoplasma-positive cells.

Basic Hoescht staining protocol

Cells were seeded into a glass microscope slide coverslip and added into each well of a clear 6-well microplate (15000 cells suspended in 500 µL of pre-warmed CCM for each well). Following that, 500 µL of 4% paraformaldehyde and the same volume of CCM to each well was added and the plate incubated for 1 hour at 37°C. Then, fixative solution was aspirated and the coverslips washed 3X 5 minutes each with pre-warmed PBS. The PBS following the final wash was aspirated. A total volume of 50 µL of Hoechst 33342, trihydrochloride, trihydrate (ThermoFisher Scientific, H3570) was added to each coverslip. The plate was then covered with foil (aluminium) and incubated at RT for 10 minutes. Coverslips were washed 3X for 5 minutes with PBS. The PBS was removed on the last wash and a drop of Fluorescence Mounting Medium (Dako®, S3023) was added to each coverslip. Thereafter, the microscope slide coverslips were mounted on the microscope slides. The microscope slides were then incubated for an hour at room temperature. The slides were viewed using the Axio Observer 7 for LSM confocal microscope (Carl Zeiss Microscopy GmbH) attached to a Colibri 7 light module (Carl Zeiss Microscopy GmbH, 5438001088) HE Dapi (358 nm) immediately or the slides was stored at -20°C, covered with foil, to view at a later stage.

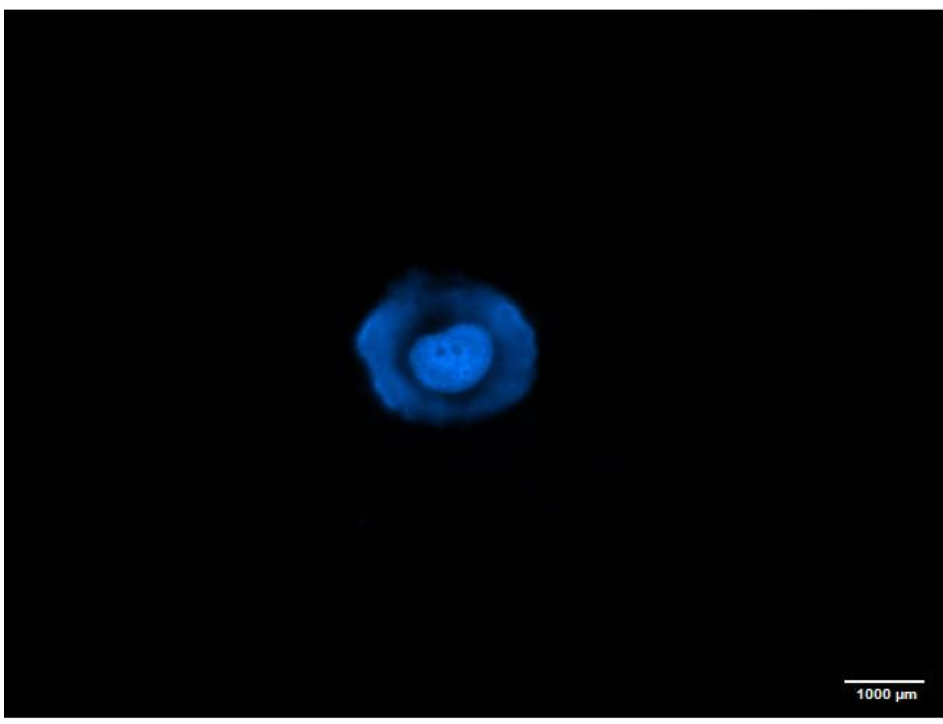

Figure 4: Fluorescent micrograph illustrating that the MCF-7 breast cancer cells utilized in this study were free of mycoplasma. Scale Bar (1000 µM) magnification (400x)

Appendix I

The MCF-7 breast cancer cells had different response to treatment following 72 hour exposure. There was a change in morphology of the cells after being exposed to the treatment groups (A) Control (normal culture media); (B) MO; (C) Dox and (D) MO + Dox

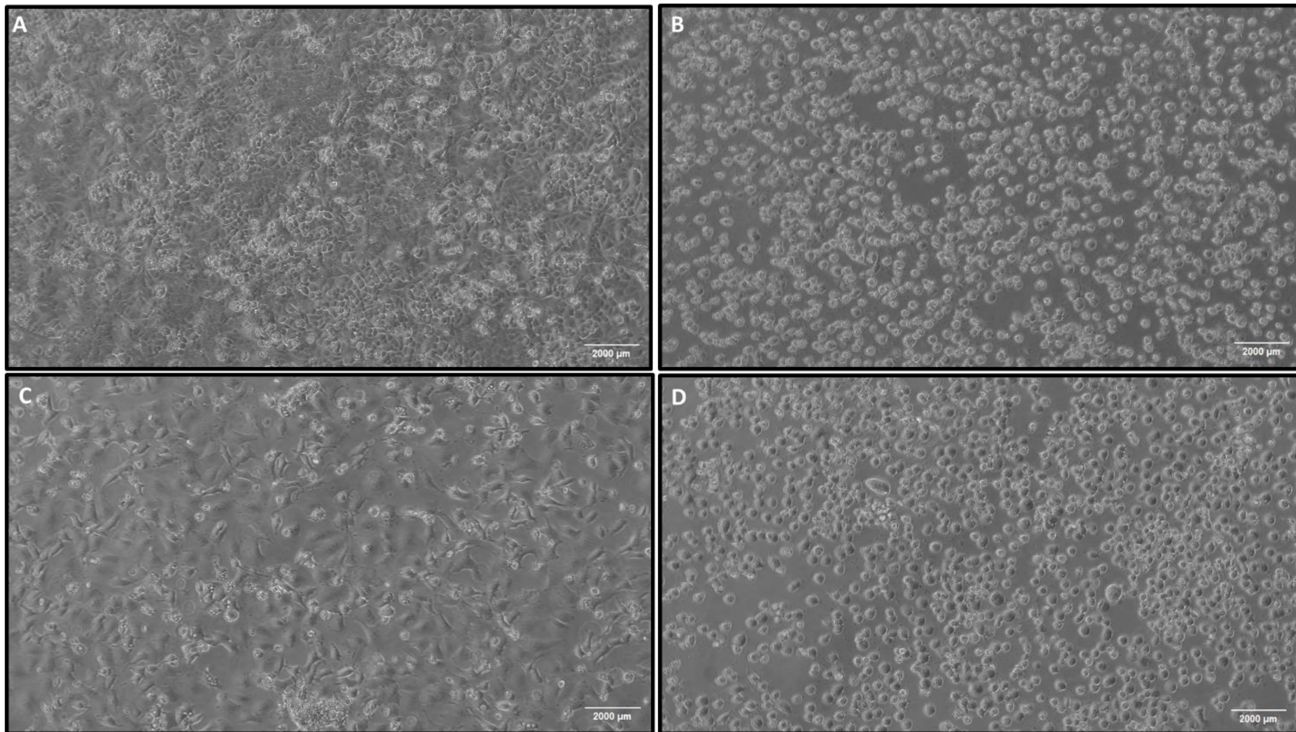

Figure 5: The MCF-7 cell morphology after 72 hours of treatment exposure. Scale Bar (2000 µM) magnification (100x)

Appendix J

LANGUAGE PRACTITIONER: Anneke-Jean Diesel

BA Communication Science (Corporate and Marketing Communications)*
BA Hons Communication Science (Corporate and Marketing Communications)*
* Cum Laude

17A Innes Avenue
Waverley, Bloemfontein

Tel: 084 244 8961
annekedenobili@gmail.com

November 2023

DECLARATION

I, Anneke-Jean Diesel, hereby declare that I did the language editing of the research of MALEBOGO M. MOREMANE (title: *Investigating the potential antiproliferative effect of Moringa oleifera aqueous leaf extract in MCF-7 breast cancer cells*). This research is submitted in fulfilment of the requirements of the degree Master of Medical Science (Physiology) in the Department of Basic Medical Sciences, School of Biomedical Sciences, Faculty of Health Sciences at the University of the Free State. All the suggested changes, including the implementation thereof, were left to the discretion of the student.

Please note:
The language editing did not include reference editing/checking or formatting. Also, the editor will not be held accountable for any later additions or changes to the document that the editor did not edit, nor if the student rejects/ignores any of the changes, suggestions or queries, which he/she is free to do. It remains the student's responsibility to ensure that the similarity index is according to the University's regulations. The editor can also not be held responsible for errors in the content of the document or whether or not the student passes or fails. It is the student's responsibility to review the edited document before submitting it for evaluation.

Sincerely

SATI Registration #: 1003466

Appendix K

List of reagents and consumables list as well as the companies they were purchased from

Chemical/Reagent	Catalogue number	Company
Cell lysis Buffer	FNN0011	Life Technologies Corporation, Carlsbad,CA, USA
Dimethyl sulfoxide (DMSO)	67-68-5	Sigma-Aldrich, St Louis, MO, USA
Dulbecco`s modified Eagle`s medium (DMEM) with phenol red	12-604F	Lonza, Walkersville, MD, USA
Fetal bovine serum (FBS)	LS-1012	Life Technology Corporation, Carlsbad,CA, USA
Penicillin-streptomycin mixture	P4333	Sigma-Aldrich, St Louis, MO, USA
L-Glutamine	, 25030149	Thermo Fisher Scientific, Waltham, MA, USA
Doxorubicin hydrochloride	D1515	Sigma-Aldrich, St Louis, MO, USA
Extra thick blot paper	1703960	Biorad, USA
Prot/Elec^{TM} tips ,	2239915	Biorad, USA

Clarity^{TM} BulkWestern ECL substrate	170-5060	Biorad,USA
Buffer 2x Laemmli (30 ml)	1610737	Biorad,USA
SDS solution 10% (250 ml)	1610416	Biorad, USA
Nitrocellulose membrane	1620117	Biorad, USA
10x Tris/Glycine buffer		Biorad, USA
10x Tris/Glycine buffer/SDS buffer	1610732	Biorad,USA
Stacking gel buffer	1610799	Biorad,USA
Resolving gel buffer	1610798	Biorad, USA
2-Mercupto ethanol	1610710	Biorad,USA
Skim mik powder	NCM0249A	Biorad,USA
Sodium Hydroxide, Pellets	3773723	Biorad, USA
2-Thiobarbituric acid	504-17-6	Biorad, USA
Paraformaldehyde	S6041115 206	Biorad,USA
Hydrochloric acid	7647-01-0	Biorad,USA
Melondialdehyde	102-52-3	Biorad, USA

Consumables

Consumables	Catalogue number	Company
0.5 mL Eppendorf safe-lock	0030 123.301	Eppendorf, Hamburg,

tubes		Germany
1.5 mL Eppendorf safe- lock tubes	0030 123.328	Eppendorf, Hamburg, Germany
2 mL Eppendorf safe- lock tubes	0030 123. 344	Eppendorf, Hamburg, Germany
15 mL centrifuge tubes	602072	SPL Life Sciences
50 mL centrifuge tubes	50050	SPL Life Sciences,
CELLBIND 96-well clear plates	3300	Corning, MA, USA
Cryotubes	430659	Corning, MA, USA
Filter Pads	23385	Sigma-Aldrich, St Louis, MO, USA
T75 Flasks	70075	SPL Life Sciences

Experimental kits

Experimental kits	Catalogue no	Company
Cell-Titer Glo Luminescent cell viability Assay	G7570	Promega, , Madison, Wilsconsin, USA
GSH-Glo^{TM} assay kit	V6911	Promega, Madison, Wilsconsin, USA
MTT assay kit	Ab211091	Abcam, Pretoria, SA
DCFH-DA assay	2439068	Cell Biolabs, San Diego,US
Caspase 3/7	G8090	Promega, Madison, Wilsconsin, USA
Caspase Glo 9 assay	G8210	Promega, Madison, Wilsconsin, USA
Caspase-Glo 8 assay	G8200	Promega, Madison, Wilsconsin, USA
High capacity RNA-to-DNA kit	4387406	Thermo Fisher Scientific, Waltham, MA, USA
BCA Protein Assay Kit	23225	Thermo Fisher Scientific, Waltham, MA, USA
Qubit DNA BR Assay kit	Q32850	Thermo Fisher Scientific, Waltham, MA, USA
Qubit RNA HS Assay kit	Q32852 & Q32855	Thermo Fisher Scientific, Waltham, MA, USA
Qubit Protein Assay kit	Q33211	Thermo Fisher Scientific, Waltham, MA, USA
Qubit 4 fluorometer system verification assay kit	Q33237	Thermo Fisher Scientific, Waltham, MA, USA
SsoAdvanced Universal SYBR green supermix	1725271	Bio-rad, USA
Water nuclease free	R0581	Thermo Fisher Scientific, Waltham, MA, USA
Qubit DNA HS Assay kit	Q33230	Thermo Fisher Scientific, Waltham, MA, USA
P53 (7F5) (Rabbit mAb)	2527	Cell signaling technology,

		Danvers, Massachusets, USA
Rb mAb to Nrf2 (EP1809Y) phospho S40	Ab76026	Cell signaling technology, Danvers, Massachusets, USA
HRP anti-beta actin antibody: Ms mAb to Beta actin (BA3R)	Ab173838	Cell signaling technology, Danvers, Massachusets, USA
Mini-protean TGX pre cast gels	4561033	Bio-rad, USA
Transblot turbo transfer pack	1704158	Bio-rad, USA
Precision protein streptactin-HRP conjugate	1610381	Bio-rad, USA
Bcl-2 (124) Mouse mAb	15071	Cell signaling technology, Danvers, Massachusets, USA
PARP-1 (46D11) (Rabbit mAb)	9532	Cell signaling technology, Danvers, Massachusets, USA
Bax (Rabbit mAb)	2772	Cell signaling technology, Danvers, Massachusets, USA

Equipment

Equipment	Product Number	Supplier
Benchtop Freeze dryer	Virtis 2KBTXL	
Agilent Bioanalyser	G2946-90004	Agilent Technologies,Santa Clara, California
CountessTM Automated Cell Counter	C10227	Invitrogen, Carlsbad, CA, USA
Benchtop Centrifuge	SL16R	Thermo Fisher Scientific, Waltham, MA, USA
Dri-Block® (Heat block)	DB200/3	Techne®
Micro-centrifuge	001977	Eppendorf, Hamburg, Germany
NanoDrop™1000 Spectrophotometer	A984	Thermo Fisher Scientific, Waltham, MA, USA
Nikon Eclipse Ti inverted microscope	Ti inverted	Nikon, Tokyo, Japan
Glomax® Discover Microplate Reader	GM3000	Promega, Madison, Wilsconsin, USA
Orbital shacker	20197	Stoval life Sciecne
Accuri™ C6 flow cytometer		BD Biosciences, USA